Systemic immune markers characterizing early stages of rheumatoid arthritis

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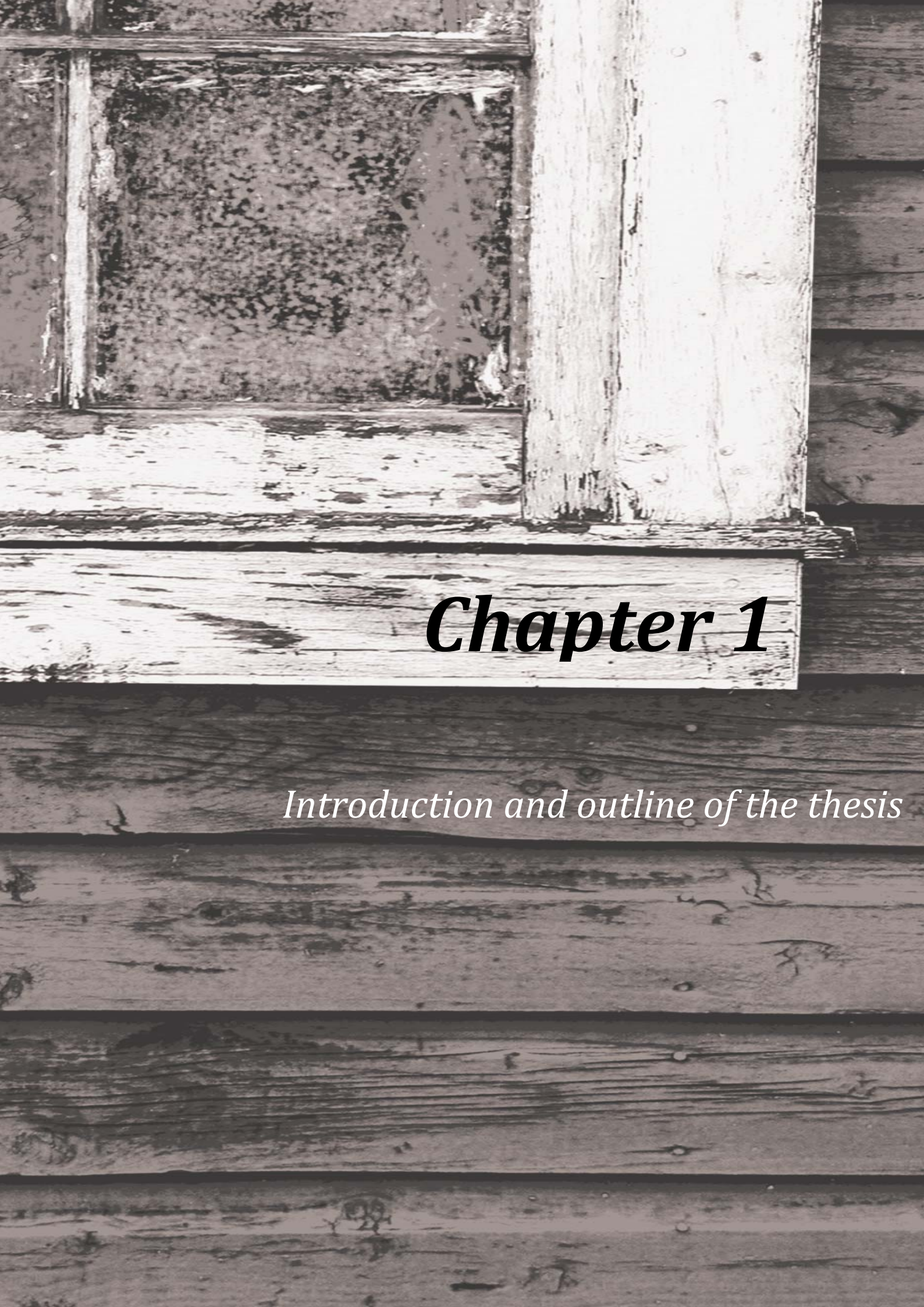
Contents



Chapter 1

Introduction and outline of the thesis

The essence of immunity is to defend against foreign matter (i.e. pathogens) while maintaining tolerance to self. This state of immune tolerance is the cornerstone of protection against autoimmune diseases. The balance between an effective immune response and its ability to timely auto-suppress such response, ensures healthy living. Among numerous factors (internal and external) that disrupt this balance, aging is considered to be important. The human body's ability to defend itself against infectious diseases, cancer and autoimmune diseases has been demonstrated to decline with age. Accordingly, development of rheumatoid arthritis (RA), one of the most common autoimmune diseases, has been associated with the aging of the immune system (1).

Numerous alterations of the immune response have been described in RA patients. These include increased expression levels of various cytokines, such as IL-1β, IL-2, IL-6, TNF-α in the peripheral blood (PB), synovial fluid (SF) and synovial tissue from RA joints (2-5). Further immune alterations attributed to RA pathology are: increased numbers of T-cells with a senescent or exhausted (terminally differentiated) phenotype (6-8); decreased numbers of circulating NK-cells (9, 10); disturbed regulatory T-cell function (11); telomere erosion of hematopoietic stem cells (12-14) and subsequent premature telomere shortening of circulating leukocytes (15, 16); expansion of monocytes (17, 18), T-cells (5, 19) and B-cells (3, 20, 21) with pro-inflammatory effector functions in PB and SF. Furthermore, RA is characterized by the presence of autoantibodies. Approximately 70% of RA patients are seropositive for autoantibodies, such as anti-cyclic citrullinated peptide antibodies (ACPA) and ~80% are seropositive for rheumatoid factor (RF) (22, 23). Seropositivity for autoantibodies (e.g. ACPA) is not only one of the diagnostic criteria for RA (24), but its presence in otherwise healthy people indicates a high risk of future RA development (25-29).

Autoantibodies are not just disease markers but may have an active, functional role in RA pathogenesis. Autoantibodies can induce pro-inflammatory cytokine production by FcγR-dependent triggering of macrophages in vitro (30-32). In the pre-clinical stage of RA, emergence of ACPA and RF as well as a broadening of the ACPA repertoire (epitope spreading) preceded the elevation of serum cytokine levels (27, 29).

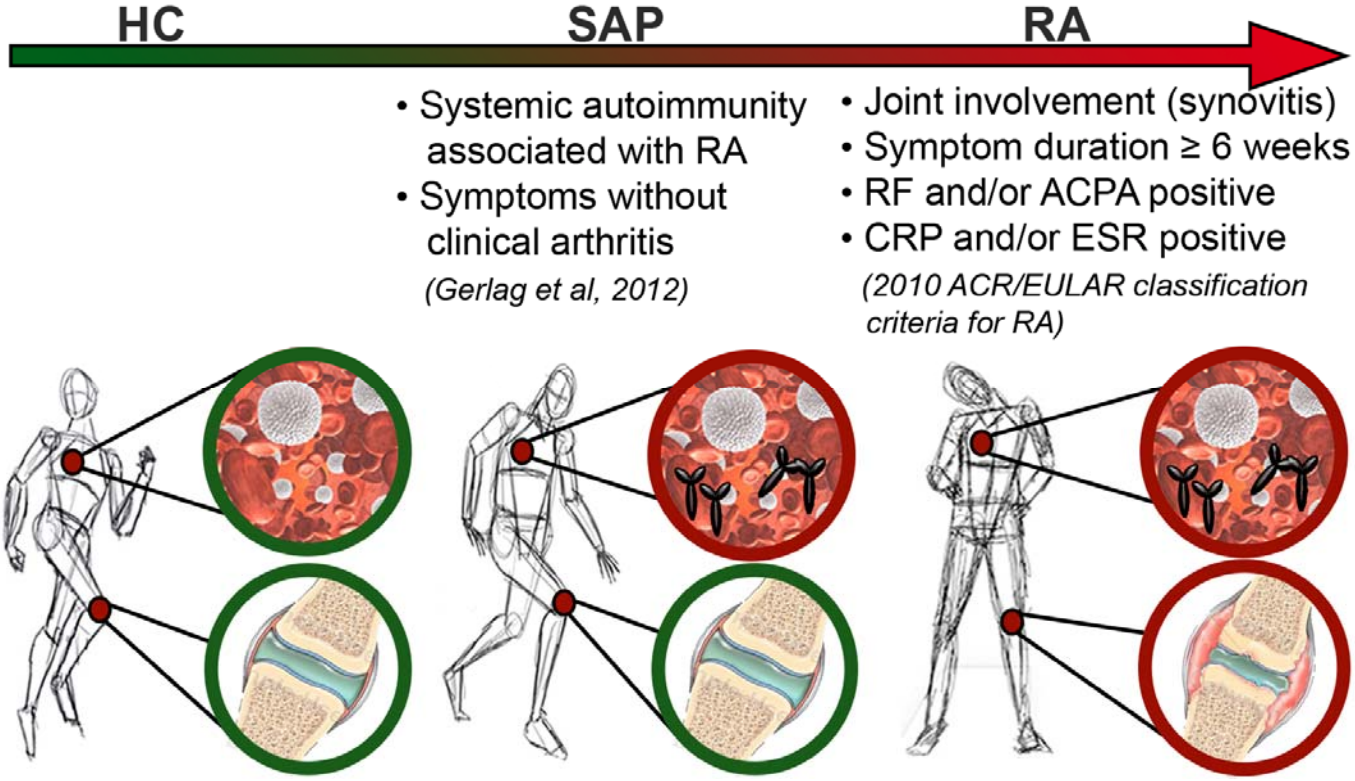
Aggressive treatment early in the disease course has been shown to lead to long-term improvement and reduction of the risk of radiographic progression (33-36). A step forward in RA research, based on the concept of the therapeutic “window of opportunity”, involves postponing/preventing the disease development by therapeutic intervention before the onset of all clinical symptoms of RA (37-39). Recent studies suggest that subjects at high risk of RA development, thus most eligible for a preventive intervention, include first-degree relatives of RA patients and seropositive arthralgia patients (SAP) (40, 41).

Inclusion of well-defined cohorts, such as SAP, in basic translational research and in clinical studies may thus likely improve our understanding of the immunopathogenesis of RA. Similarly important cis the inclusion of RA patients who have only recently been diagnosed with RA and who are not yet treated

with disease modifying anti-rheumatic drugs (DMARDs, second-line treatment), corticosteroids or biologics (third-line treatment). This approach may allow to identify immune defects primarily involved in the disease, and to avoid focusing attention on changes only secondary to a long-term inflammatory process. It also excludes the possibility that the observed immune alterations (or the lack thereof) are a consequence of the drug's effects (or the drug-induced normalization of the primary immune defects).

Our overall goal was to identify systemic immune markers characterizing early stages of RA, as these may provide clues as to the primary factors involved in the development of a pathologic autoimmune response. Therefore, in all our studies, patients at the very early stage of the disease - newly diagnosed with RA, DMARD/glucocorticoids/biologics-free as well as seropositive arthralgia patients (SAP) were asked to participate. SAP are at high risk of developing RA (41). According to the European League Against Rheumatism (EULAR) recommendations, this risk is based on the presence of systemic autoimmunity associated with RA and symptoms without clinical arthritis (42) (**Fig. 1**). Similar to newly diagnosed RA, immune alterations occurring in SAP may represent primary defects involved in the pathogenesis of RA, rather than secondary effects of chronic immune stimulation as seen in later phases of RA.

Figure 1. Depiction of the cohorts, as well as body compartments: peripheral blood (indicated with upper circles) and joints (indicated with lower circles) studied.



Some of the immune markers investigated in our cohorts, were previously described in long-standing, treated RA only.

In order to elucidate a role of autoantibodies in inflammatory processes, early RA cohorts were stratified according to ACPA/RF status, into seropositive (ACPA+ and/or RF+) and seronegative (ACPA- and RF-).
In the studies reported in this thesis, we focused primarily on the periphery, while the local inflammatory sites (synovial fluid and tissue) were studied only in late- stage RA (**Fig. 1**). This approach stems from the notion that RA does not begin at the level of the joint but rather that systemic inflammation precedes synovitis (29). In line with this notion, recent studies showed no evidence for the presence of subclinical synovitis in SAP (43, 44).

The main objective of our studies was to identify immune alterations that may play a role in RA onset:

In **Chapter 2**, in a comprehensive review, we have summarized and evaluated published data on the role of immunosenescence in rheumatoid arthritis. We addressed the still open question whether premature immunosenescence in RA is the primary, genetically determined factor underlying the disease or whether it is a secondary consequence of the already ongoing RA-associated inflammation. It could be concluded that convincing evidence necessary to answer this question, is still lacking. Therefore, suggestions for future (longitudinal) studies in well defined patient's cohorts are provided.

In **Chapter 3** the expression of various serum immune markers (cytokines, chemokines, cytokine receptors) in samples obtained from SAP, seropositive RA (SP RA) and seronegative RA (SN RA) compared to healthy controls, was analyzed. The aims of the study were to identify markers discriminating between SP and SN RA and markers identifying SAP at high risk of RA development.

Although NK cells have various immunomodulatory capacities, the role of NK-cells in the immunopathogenesis of RA is unclear. Therefore, in **Chapter 4**, we investigated causes and consequences of alterations of NK-cell subsets in the early stages of RA development.

In **Chapter 5** the pathogenic role of CD4+ T-cells expressing CD161; recently identified as a common marker of Th17 progeny; in SAP, early RA and late RA patients was assessed. Our findings of a pro-inflammatory role of CD4+CD161+ T-cells primarily in the inflamed synovium, prompted the study described in **Chapter 6**. In this study, expression of lectin-like transcript 1 (LLT1), representing the endogenous ligand for CD161, in RA joints and in the periphery was investigated.

Previously, we reported that CD70-expressing CD4+ T cells are enriched in RA and promote autoimmunity via IFN-γ and IL-17 expression (45). In **Chapter 7** we studied if CD70-expressing T-cells are modulated in the different phases of RA development. Also, we studied the dynamics of CD70 expression regulation in vitro.

Chapter 8 discusses the role of immunosenescence in the progression to chronic inflammation, which is characteristic for autoimmune-mediated inflammatory diseases (AIMIDs), such as RA.

Finally, in **Chapter 9** we present an overview of our findings on immune marks in the different stages of disease. We discuss the implications of our findings for the current understanding of the RA disease process and for early recognition of the at-risk subjects. Also, directions for future research are proposed.

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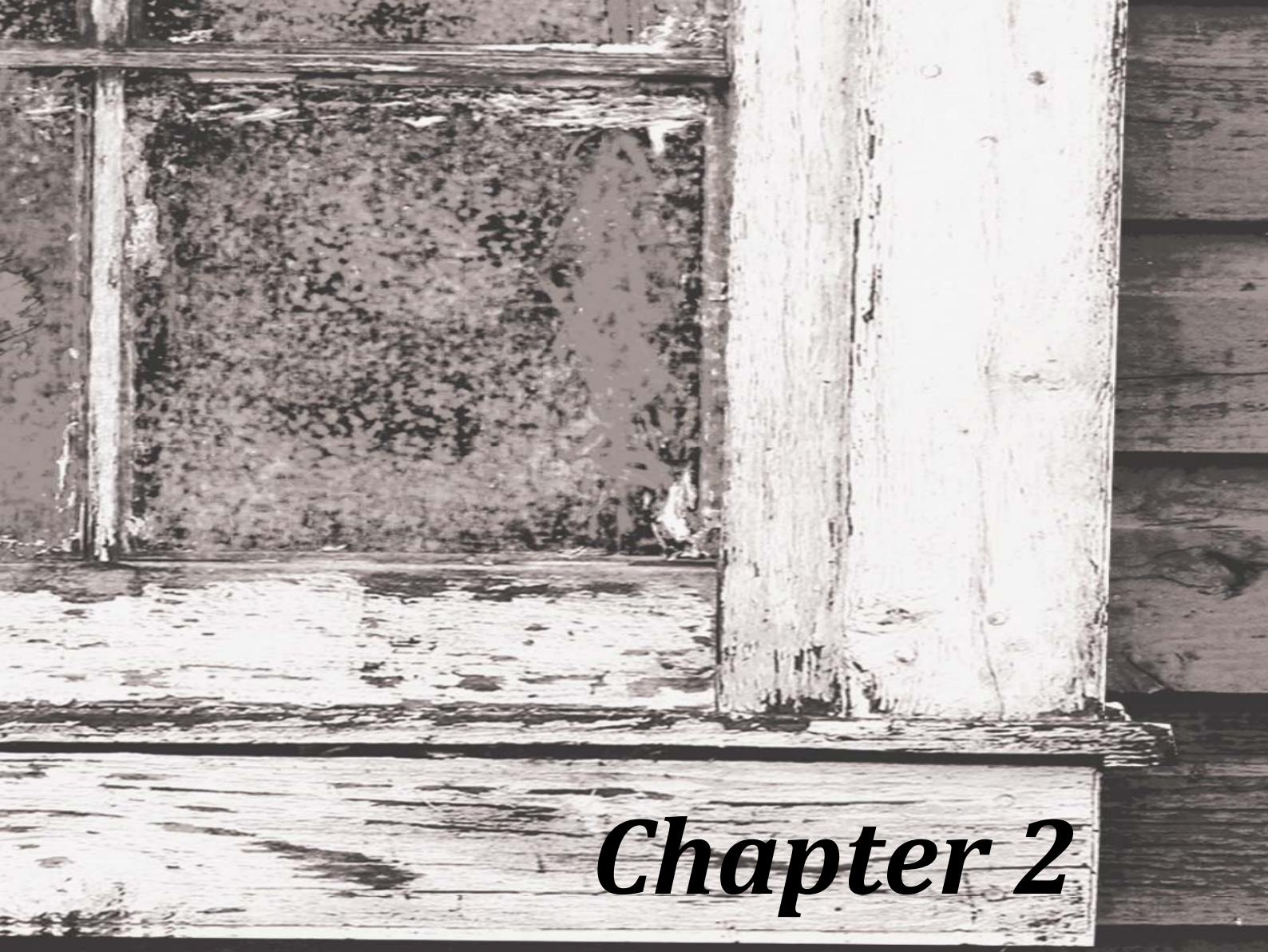

Chapter 2

Rheumatoid arthritis, immunosenescence and the hallmarks of aging

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Abstract

Age is the most important risk factor for the development of infectious diseases, cancer and chronic inflammatory diseases including rheumatoid arthritis (RA). The very act of living causes damage to cells. A network of molecular, cellular and physiological maintenance and repair systems creates a buffering capacity against these damages. Aging leads to progressive shrinkage of the buffering capacity and increases vulnerability. In order to better understand the complex mammalian aging processes, nine hallmarks of aging and their interrelatedness were recently put forward.

RA is a chronic autoimmune disease affecting the joints. Although RA may develop at a young age, the incidence of RA increases with age. It has been suggested that RA may develop as a consequence of premature aging (immunosenescence) of the immune system. Alternatively, premature aging may be the consequence of the inflammatory state in RA. In an effort to answer this chicken and egg conundrum, we here outline and discuss the nine hallmarks of aging, their contribution to the pre-aged phenotype and the effects of treatment on the reversibility of immunosenescence in RA.

1. Introduction

Age is the most important risk factor for development of chronic inflammatory diseases including rheumatoid arthritis (RA). Aging is a complex process, which is not yet fully understood. In order to better understand the complex mammalian aging processes, nine hallmarks of aging and their interrelatedness were recently put forward. In essence, primary hallmarks involve acquisition of cell biological forms of damage such as genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis. Secondary hallmarks involve antagonistic, compensatory responses to these damages such as deregulated nutrient-sensing, mitochondrial dysfunction and cellular senescence. Lastly, the integrative hallmarks represent the result of the primary and secondary events such as stem cell exhaustion and inflammaging, leading to deterioration of cellular and organismal function as seen with aging (1).

Age-associated changes in immune function termed immunosenescence are thought to be responsible for the increased morbidity with age. Both the innate and the adaptive arm of the immune system undergo marked changes with age and contribute to the process of immunosenescence (2). With age, innate immune mechanisms generally become more active, whereas functioning of the adaptive immune system generally declines.

Immunosenescence is characterized by i) thymic involution leading to a steady decline in the production of naïve T-cells, ii) shrinkage of the T-cell repertoire through continuous antigen stimulation favoring the development of functionally altered, oligoclonal, senescent T-cells identified by CD28 loss and iii) a chronic low degree of inflammation termed inflammaging as evidenced by increased serum levels of inflammatory cytokines such as TNF-α, IL-6 and acute phase proteins (3). These changes in cellular composition and cellular functions create a pro-inflammatory environment which might accelerate development of RA.

RA is a chronic auto-inflammatory disorder targeting the joints. Although RA can develop in individuals of any age, its incidence continues to increase with age into the seventh decade (4). When compared to healthy individuals of the same age, RA patients show significantly more prominent features of immune system aging. Despite the growing experimental data, the question whether accelerated immunosenescence is a primary cause of RA or an event secondary to the chronic inflammatory process, remains to be answered.

In this review we summarize the experimental evidence for the pre-aged phenotype of different immune cells in RA and their relevance to disease pathogenesis. We also compare features of senescence in peripheral versus local tissues (bone marrow, peripheral blood and the inflamed joint). Furthermore, we outline the contribution of the hallmarks of aging to the pre-aged phenotype in RA. Also, the effects of treatment on the reversibility of immunosenescence in RA are discussed. Lastly, novel insights in the molecular pathogenesis of RA relevant to the development of the pre-aged phenotype may guide adequate design of conclusive patient-based research to solve this issue.

2. Evidence for a pre-aged phenotype in RA

2.1. Immunosenescence and T lymphocytes in RA

Although aging affects all cells of the immune system, T-cells appear to be most sensitive. A prominent feature of T-cell aging is the oligoclonal expansion of CD4+ and especially CD8+ T-cells lacking expression of the co-stimulatory molecule CD28. CD28 co-stimulation, required for efficient T-cell activation and proliferation, is progressively lost with age (3). Consequences of CD28 loss differ between CD4+ and CD8+ T-cells; in CD8+ T-cells loss of CD28 can lead to dysfunction or towards a regulatory phenotype, whereas in CD4+ T-cells CD28 deficiency is associated with acquisition of novel NK-like functionalities. Loss of CD28 in CD4+ T-cells is associated with an increased production of pro-inflammatory cytokines, increased cytotoxicity, via expression of perforin and granzyme B and a propensity to migrate into tissues. Interestingly, CD4+CD28- T-cells were found expanded in patients with several chronic autoimmune conditions, including RA. Expansions were seen in both early and late RA patients and were more prominent in carriers of the RA-susceptibility HLA-DRB1*04 alleles (5). This suggested that accelerated immunosenescence is a genetically-driven phenomenon that might be causal to RA development rather than the consequence of disease.

Novel functional features of senescent CD28- T-cells have been associated with phenotypical changes, such as *de novo* expression of NK receptors: Immunoglobulin (Ig)-like (i.e. killer cell activating receptors (KAR), killer cell inhibitory receptors (KIR)) (6-8) and C-type lectin-like superfamily (NKG2D) receptors (9,10). KIRs and KARs are specific for classical MHC class I molecules (HLA-A, HLA-B, HLA-C) and the non-classical MHC molecule HLA-G (11). Ligands for NKG2D include the stress-induced MHC class I polypeptide-related sequence (MIC) proteins. MIC proteins were found upregulated by RA synoviocytes (10). Also, CD28- T-cells in RA patients demonstrated increased expression of NK cell-associated receptors CD56 (9,12,13) and CD57 (14).

Upregulation of NK receptors likely serves a co-stimulatory role in CD28- T-cells. Cross-linking of KAR in the presence of anti-CD3 induced proliferation of CD4+CD28- T-cells (6). Engagement of NKG2D, in the presence of anti-CD3, induced proliferation, IFN-γ and TNF-α secretion (10). Triggering of CD56 alone led to the production of IL-2, TNF-α and MIP-1β (12). Furthermore, several studies showed upregulation of CD70, a member of the TNF superfamily, by CD28- T-cells (15-17). CD70 expression lowered activation threshold of CD28- T-cells (15). In line with their pro-inflammatory potential, RA patient-derived peripheral blood (PB) CD4+CD28- T-cells (14,18,19) and CD4+CD28- T-cell clones (14) produced significantly higher levels of TNF-α and IFN-γ than their CD28+ counterparts.

The exact role of senescent T-cells in the clinical course of RA is still unclear. Numbers of circulating CD4+CD28- T-cells did not correlate with RA clinical parameters such as C-reactive protein (CRP), level of anti-cyclic citrullinated protein antibodies (ACPA), swollen and tender joint counts or disease duration (14,20,21). Some studies demonstrated a correlation between CD4+CD28- T-cells and erosions in RA

(22,23) but this was not confirmed by others (21,24). Expansions of CD4+CD28- T-cells in RA have been implicated in the development of atherosclerotic disease (24) and in extra-articular manifestations (21,24).

The role of senescent T-cells at the level of the joint in RA also remains unclear. Several studies demonstrated that CD4+CD28- T-cells are less frequent in RA synovial fluid (SF) or synovium than in PB (14,18,20,25). This seems to contradict the tissue tracking propensity of CD4+CD28- cells suggested based on the expression of adhesion molecules facilitating tissue infiltration (CD11a, CD49d) (9,18,26) and receptors (NKG2D, CX_3CR1) which ligands (MIC, fractalkine) are readily expressed in RA tissue (9,10). A possible explanation may involve the restoration of CD28 expression by IL-12 (27) which is abundantly expressed in RA joints (28). Consistent with this explanation, several similarities between PB CD28- and SF CD28+CD4+ T-cells have been demonstrated. These include the methylation status of IFN-γ promoter, expression of CXCR3, CCR6, CCR7 (19) and clonotypic composition (25).

Several studies highlighted the importance of CX_3CR1, a chemokine receptor exclusively expressed by CD4+CD28- T-cells (9,29,30) and its ligand fractalkine (FKN) expressed by fibroblast-like synoviocytes (FLS), as co-stimulatory pair relevant to the local inflammatory process. The CX_3CR1-FKN interaction between CD4+CD28- T-cells and FLS induced expression of pro-inflammatory cytokines and relayed survival signals. Furthermore, the CX_3CR1-FKN interaction facilitated proliferation of FLS (29,30).

It has been suggested that CD4+CD28- T-cells in RA are autoreactive. So far, conclusive evidence for this notion is lacking. RA-derived CD4+CD28- T-cell clones were shown to proliferate in response to autologous adherent cells *in vitro* (25), while CD4+CD28-NKG2D+ T-cell clones secreted IFN-γ when cultured with autologous MIC+ synoviocytes (10). CD4+CD28- T-cells did not respond to collagen type II (18), a putative RA-associated autoantigen. In contrast, the reactivity of CD4+CD28- T-cells towards CMV has been clearly demonstrated, and did not differ between RA- and healthy control (HC)-derived or RA- and multiple sclerosis-derived senescent T-cells (18,31,32). Thus, clonally expanded CD4+CD28- T-cells in RA are likely CMV-specific but may also include selfreactive clones (31). Senescence features reported in various stages of T-cell development in RA are depicted in the Figure.

2.2. CMV as accelerator of T-cell immunosenescence

CMV infection has been demonstrated to drive immunosenescence directly, by imposing chronic replicative stress and indirectly, by induction of IFN-α expression by plasmacytoid dendritic cells (PDC). IFN-α has been shown to accelerate differentiation and telomere shortening through inhibition of telomerase activity (33).

Latent CMV infection has been linked to a pro-inflammatory state as evidenced by increased systemic levels of IL-6 and TNF-α (34). TNF-α is known to downregulate CD28 expression (35). Latent CMV infection was found to aggravate the clinical course of RA (36). CMV-specific CD4+CD28- T-cells were

found increased in CMV+ but not CMV- RA patients (20). These cells represented potent producers of IFN-γ (20,36,37).

Despite its role in the development of an immunosenescent phenotype, numerous studies undermine the notion of CMV involvement in RA pathogenesis. The most obvious, epidemiological evidence shows a worldwide CMV prevalence of 40-99%, while only ~1% of all individuals develop RA. A study by Pierer *et al*, which included large cohorts (>200 subjects each) of RA patients and HC, showed similar prevalence of CMV infection as well as comparable titers of anti-CMV antibodies in these groups (36).

Presence of CMV-specific T-cells, CMV DNA or CMV early antigen protein has been demonstrated in RA synovial tissue or synovial fluid but also at inflammatory sites of other autoimmune conditions (38-43).

In conclusion, CMV-driven T-cell immunosenescence is well established in both HC and in RA patients. The data on CD28- T-cells imply that these cells contribute to the inflammatory milieu in several chronic inflammatory conditions including RA. Also, CD28- cells may be involved in (extraarticular) tissue injury. Their contribution to joint pathology is hard to establish, partly because cells may regain CD28 expression via an IL-12 dependent mechanism at the level of the joint.

2.3. Immunosenescence and B lymphocytes in RA

B-cells play an important role in RA pathogenesis based on their ability to produce and secrete autoantibodies and their role as antigen presenting cells (44-47). Aging has pronounced effects on both numbers and functions of B-cells. Available evidence shows a decline of PB B-cells with age (48). Yet, peripheral B-cell numbers were not further decreased in newly-diagnosed RA patients when compared to age-matched controls (49).

To the best of our knowledge, no studies have elucidated whether B-cells from RA patients show features of premature senescence. Recently, Rubtsov *et al* identified a novel population of age-associated B-cells (ABCs), defined as CD19+CD11b+CD11c+, in aged female mice. ABCs were significantly increased in autoimmune-prone mouse strains at the onset of autoimmune disease and were found to be the main source of autoantibody production. The human equivalent of mouse ABCs, defined as CD19+CD11c+CD21- B-cells, were identified in the PB of some elderly female patients with RA (50). Further studies are required to confirm the accumulation and functional role for ABCs in RA pathogenesis. Besides the increase of potentially pathogenic B-cells, a decrease of IL-10 producing B-cells may contribute to RA development. Both the number and function of immature transitional B-cells (with CD19+CD24^{high}CD38^{high} phenotype) regarded as the main IL-10 producers among B-cells, were found to decrease with age. The frequency of IL-10-expressing cells among CD24^{high}CD38^{high} B-cells was negatively correlated with rheumatoid factor (RF) titers (51). In conclusion, although it is evident that aging and inflammation affect B-cell numbers and functional subsets, there is no sound evidence to support a role of senescent B-cells in RA pathogenesis.

2.4. Immunosenescence and NK-cells in RA

Aging-associated numerical, phenotypical and functional changes in NK-cells have been comprehensively reviewed (52-55). Healthy aging is associated with an increase in the number of NK-cells, mainly attributed to the expansion of the differentiated, mature $CD56^{dim}$ subset that develops from the immature $CD56^{bright}$ subset (56). $CD56^{bright}$ NK-cells were reported to either decrease (57,58) or remain stable with age (56). While NK-cell cytotoxicity is well-preserved with age (57), the cytokine and chemokine expression pattern of NK-cells, their proliferative capacity and their responsiveness to cytokines were impaired upon aging (56,57,59).

In contrast to healthy elderly, RA patients are characterized by a decline of peripheral NK-cell number (60-62). A decline of these cells may accelerate senescence in RA patients. Support for this notion comes from studies demonstrating that NK-cells clear senescent cells in tumor lesions (63) and eliminate senescent cells involved in tissue damage (64). These data indicate an immunosurveillant role of NK-cells in RA senescence rather than a pro-inflammatory role.

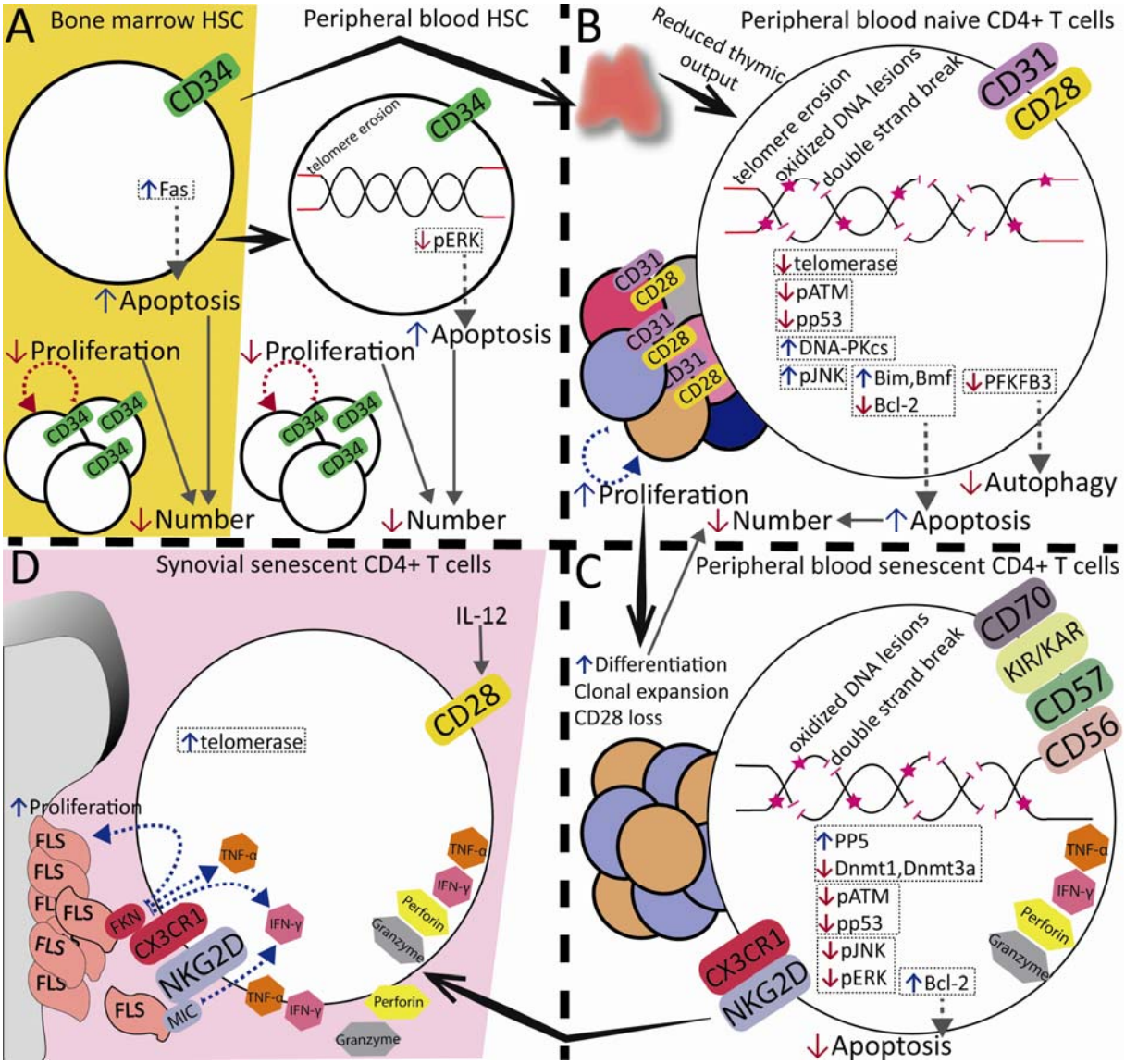
2.5. Immunosenescence and monocytes in RA

Three different monocyte subsets can be distinguished based on surface expression of CD14 and CD16 (65,66), namely the classical monocytes ($CD14^{bright}$ CD16-), the intermediate monocytes ($CD14^{bright}$ CD16+) and the non-classical monocytes ($CD14^{dim}CD16^{bright}$). The latter subset has been suggested to represent senescent monocytes, due to a shorter telomere length compared to classical monocytes and expression of the senescence-associated β-galactosidase (SA-βgal) (67,68). The $CD14^{dim}CD16^{bright}$ non-classical monocytes are also more pro-inflammatory and express chemokine receptors facilitating migration to tissues at significantly higher levels than classical monocytes (67,69). $CD14^{dim}CD16^{bright}$ monocytes were found to be similarly increased in elderly individuals with atherosclerosis and RA patients when compared to young subjects (67,70).

Another population of monocytes that was increased with age is the CD56 NK receptor-expressing monocyte subset. These $CD14^{bright}$CD56+ monocytes were found to be potent producers of cytokines and reactive oxygen species (ROS). Interestingly, only young RA patients (<40 years) showed $CD14^{bright}$CD56+ monocyte expansion when compared to HC. As the number of $CD14^{bright}$CD56+ monocytes did not correlate with disease duration, medication or C-reactive protein levels, the cause of the reported increase in these young RA patients remains unclear (71). More studies are needed to elucidate the nature of senescence-associated changes of monocyte populations and to establish whether these alterations are more profound in RA patients than in aging *per se*.

In conclusion, ample evidence supports a pre-aged phenotype in RA patients within the T-cell compartment, whereas for other PB cells this is less evident. The culprit phenotype is represented by late stage, pro-inflammatory T-cells that have lost CD28 expression. Senescent cells may thrive because NK-cell surveillance is significantly reduced in RA.

Figure 1. Depiction of senescence-associated alterations reported within A) hematopoietic stem cells (HSC) in bone marrow and peripheral blood, B) naive CD4+ T-cells in peripheral blood, C) senescent (CD28-) CD4+ T-cells in peripheral blood and D) senescent (CD28-) CD4+ T-cells in the inflamed joint of RA patients.



A) In RA, bone marrow-derived hematopoietic stem cells (defined as CD34+) show impaired proliferative capacity and increased expression of Fas, rendering them apoptosis-sensitive. Consequently, RA patients show reduced HSC numbers at the level of the bone marrow. Peripheral blood-derived CD34+ HSC show similar defects in proliferation and increased susceptibility to apoptosis. Increased apoptosis sensitivity is via impaired ERK pathway signaling and/or age-inappropriate telomere erosion of CD34+ HSC in peripheral blood.

B) Reduced thymic output in RA was evidenced by reductions of circulating recent thymic emigrants (defined as CD31+ or T-cell receptor excision circle (TREC)+ T-cells). This is explained by inadequate supply of HSC from the bone marrow and/or age-inappropriate enhanced thymic atrophy. Peripheral blood naive T-cells (defined as CD31+CD28+) are characterized by telomere shortening, increased levels of oxidized lesions and double-strand DNA breaks, decreased telomerase activity, deficiency of proteins involved in the DNA damage response (phosphorylated forms of ATM and downstream p53), overactivation of DNA-PKCs-JNK pathway, overexpression of proapoptotic proteins Bim, Bmf and downregulation of antiapoptotic protein Bcl-2. Defects of naive CD4+ T-cells may facilitate their accelerated differentiation towards the senescent state, when subjected to proliferative stress and enhance apoptosis.

C) Compensatory hyperproliferation of the naive CD4+ T-cell pool leads to the expansion of senescent cells (characterized by the loss of CD28, de novo expression of NK receptors (KIR/KAR, CD56, NKG2D), upregulation of CD57, CD70, CX3CR1, expression of IFN-γ, TNF-α and cytotoxic molecules). Senescent CD4+ T-cells show further increase of DNA damage (double-strand breaks, oxidized DNA lesions and diminished levels of ATM and p53 phosphorylation) relative to naive CD4+ T-cells. Diminished activity of JNK and ERK pathways is associated with the increased expression of anti-apoptotic protein Bcl-2 and concomitant resistance to apoptosis of senescent CD4+ T-cells.

D) Expression of CX3CR1 facilitates migration of senescent CD4+ T-cells towards soluble Fractalkine (FKN) abundant at the level of synovium. Interaction with FKN expressed on the surface of fibroblast-like synoviocytes (FLS) augments expression of IFN-γ, TNF-α and stimulates release of cytotoxic granules by senescent T-cells and enhances proliferation and FKN expression by FLS. IFN-γ production is also promoted by the interaction of NKG2D with FLS-expressed MIC. IL-12 increased at the level of inflamed joints, may restore CD28 expression by senescent T-cells.

3. The nine hallmarks of aging and RA

Nine common denominators of aging have recently been proposed (1). The aim of this paragraph is to summarize the available evidence for the presence of these senescence hallmarks in the context of RA.

3.1. Genomic instability

Cellular senescence or stable cell cycle arrest can be either telomere-dependent (discussed in "3.2. Telomere shortening") or telomere-independent. The latter develops as a consequence of DNA damage accumulation due to replication errors, ROS, genotoxic drugs or UV light. Excessive DNA damage (including telomere erosion) evokes a persistent DNA damage response (DDR) which induces senescence or programmed cell death, both representing means to prevent malignant transformation. Factors involved in cell fate determination are not known but may include the extent of DNA damage, the strength and duration of DDR signaling and the type of the affected cell (72-74).

Elevated levels of DNA double-strand breaks were demonstrated in PB mononuclear cells (PBMC) (75,76), or isolated naïve and memory CD4+ T lymphocytes (77,78) but not neutrophils (79) from RA patients compared to HC. Similarly increased levels of DNA double-strand breaks were seen in naïve CD4+ T-cells from treatment-naïve recently diagnosed RA patients (77). Also, increased levels of mutagenic DNA adducts such as 8-oxo-guanine (8-oxo-7-hydrodeoxyguanosine or 8-hydroxyguanine) or Heptanone-Etheno-2'-Deoxycytidine (HεdC) were found. 8-oxo-guanine and HεdC represent markers of oxidative DNA damage. In RA, levels of 8-oxo-dG and HεdC were found significantly increased within DNA derived from urine (80), whole blood cells (81), PBMC (75), CD4+ T-cells (77) and naïve CD4+ T-cells (78).

Accumulation of DNA damage in T-cells of RA patients has been associated with defects in DNA repair mechanisms. First evidence came from studies demonstrating an impaired ability of RA patient-derived PBMC to repair the mutagenic base lesion O^6-methylguanine induced by the methylating carcinogen N-methyl-N-nitrosourea (82-84). Also, a decreased rate of repairing double-strand DNA breaks by RA-derived PBMC has been demonstrated using the DNA unwinding in alkaline solution assay (76). Studies by Shao *et al* demonstrated accumulated DNA damage in both naïve and memory T-cells from newly-diagnosed RA patients and related this to decreased levels of the DNA repair kinase ataxia telangiectasia mutated (ATM). ATM deficiency was shown to recapitulate the pre-aged phenotype as seen in RA T-cells whereas overexpression of ATM reconstituted DNA repair capabilities (77,78). Thus, both the increase in DNA damage-inducing events such as replicative and oxidative stress as well as the impaired

levels of proteins involved in DNA repair are likely the cause of DNA damage accumulation and permanent growth arrest of RA T-cells.

3.2. Telomere shortening

Telomere erosion is recognized by the cell as a persistent DNA damage (85-87). Telomere-dependent cell cycle arrest is mediated by upregulation of the p53-dependent DNA damage pathway (88). Telomeres shorten 50-100 bp after each replication cycle, due to the end-replication problem of DNA polymerase (85,88). Activation of telomerase prevents replicative senescence by maintaining telomere function. Telomerase is a ribonucleoprotein complex consisting of the catalytic unit called the telomerase reverse transcriptase (hTERT) and telomerase RNA (TERC). Elongation of telomeres occurs through *de novo* reverse transcription. Expression of hTERT is a limiting factor for telomerase activity. In healthy conditions, telomerase is active only in stem cells and activated T and B lymphocytes (89).

Compared to age-matched HC, RA patients showed enhanced telomere shortening in granulocytes (5), PBMC (90) and CD4+ T-cells (5). The latter was attributed to telomere erosion of naive, but not memory CD4+ T-cells by Koetz *et al* (91). Similarly, telomerase activity following TCR-dependent stimulation was found to be significantly decreased in naive, but not memory, CD4+ T-cells. hTERT deficiency was associated with a reduced proliferative capacity and a higher apoptosis rate of naïve T-cells. In contrast, lymphocytes infiltrating the synovium were characterized by high telomerase activity, indicative of their activated status. The telomerase activity levels of infiltrating cells were correlated with intensity of synovial lining hyperplasia, suggesting active lymphocyte involvement in joint destruction (92). Interestingly, telomerase activity of anti-CD3 stimulated PBMC was similarly decreased in patients with early RA, multiple sclerosis and patients with flu-like symptoms (93), indicating lack of RA-specificity of telomerase insufficiency.

3.3. Changes in gene regulation

Aging is associated with alterations of epigenetic processes. These include mechanisms involved in gene regulation at the transcriptional level, i.e. histone modifications (acetylation and methylation), DNA methylation, chromatin remodeling and mechanisms involved in post-transcriptional gene regulation, i.e. non-coding (nc)RNAs expression (1). A growing body of data demonstrates a substantial role of environmental factors in modulation of the epigenome (94). Low RA concordance rates between monozygotic twins (~15%) (95-98), suggest a high relevance of the interplay between genetics and environmentally-influenced epigenetic alterations in RA pathogenesis.

Epigenetic changes are characteristic of RA synoviocytes. Reported epigenetic alterations include global hypomethylation (99,100), local hypomethylation of LINE-1 and DR-3 promoters (101,102), increased HDAC activity (103,104), Sirt1 overexpression (105) and hyperacetylation (104), local H4 acetylation within MMP-1 promoter (106), sumoylation (107), and distinct pattern of microRNA expression (108).

3.3.1. Histone modifications

One of the age-associated post-transcriptional (epigenetic) modifications include histone acetylation (1). Histone acetylation at lysine residues, mediated by histone acetyltransferases (HAT), leads to decreased levels of chromatin condensation, allowing recruitment of the transcriptional machinery and ensuing gene transciption. Inhibition of gene expression, by removal of acetyl groups, is mediated by histone deacetylases (HDAC) (109). Aging has been associated with a general increase of the histone acetylation status, as evidenced by increased H4K16 acetylation and decreased expression of the class III HDAC (NAD-dependent protein deacetylases sirtuins (Sirt)) (1).

An *in vitro* study employing RA synovial fibroblasts demonstrated a TNF-α-induced increase in HDAC and Sirt1 activity, suggesting that altered histone acetylation may be a feature of inflammation (110,111). Moreover, RA patient derived PBMC demonstrated increased HDAC activity (112), but no specific change of Sirt1 activity (105). The current available data suggest that histone acetylation is differently modulated in aging and RA.

3.3.2. DNA methylation

Chromatin methylation changes the chromatin structure to a more compact and less easily accessible state for transcription factors. Depending on the gene and cell type, both an age-associated decrease or increase in methylation status may occur. Decreased methylation status leads to enhanced mRNA and protein expression (113,114). Methylation occurs at deoxycytosine, primarily within CpG islands (repeated CpG sequences) which are associated with ~70% of the promotors in the vertebrate genome. Age-associated methylation changes have also been found to affect genes lacking CpG islands, i.e. CD11a (LFA-1) (115). Methylation is mediated by DNA methyltransferase Dnmt1, involved in the preservation of the methylation pattern after each cell division, and methyltransferases Dnmt3a and Dnmt3b, which mediate methylation of previously unmethylated DNA (116). Both Dnmt1 and Dnmt3a expression generally decrease with age (114).

Decreased methylation status has been reported in aging cells of various types, including fibroblasts (117) and stem cells (118). Hypomethylation of a single CpG in the IL-6 promoter region was found more frequent in RA PBMC when compared to HC PBMC. High levels of IL-6 mRNA in LPS-stimulated macrophages were associated with this single CpG hypomethylation (119). The most apparent link between hypomethylation and autoimmunity, however, is constituted by the demonstration that functional alterations in senescent CD4+CD28- T-cells are brought about by changes in DNA methylation. Relative to the CD4+CD28+ T-cell population, Dnmt1 and Dnmt3a levels and the DNA methylation status were significantly decreased in senescent CD4+CD28- T-cells. Genes overexpressed in response to hypomethylation included those associated with effector functions of senescent cells, i.e. IFN-γ (19) CD70, KIR2DL4 and perforin (16,17). Decreased levels of Dnmt1 and Dnmt3 in RA CD4+CD28- T-cells are the consequence of impaired signaling of the ERK and JNK pathways. Similar defects have been noted in CD4+CD28- T-cells generated *in vitro* by repeated stimulation as wells as in CD4+CD28- T-

cells from elderly subjects (17). Interestingly, SF-derived CD4+ T-cells showed prominent hypomethylation of the IFN-γ promoter (IFNG), irrespective of CD28 expression (19).

3.3.3.Chromatin remodeling

Chromatin remodeling is mediated by enzymes involved in DNA and histone post-transcriptional modifications as well as Heterochromatin Protein 1α (HP1α), Polycomb Group (PcG) or the Nucleosome Remodeling and Deacetylase (NuRD) protein complexes. Their expression levels decrease during aging (1). RA-derived synovial fibroblasts showed altered levels of the PcG protein EZH2 (120).

3.3.4. ncRNAs

The best studied group of ncRNAs in both health and RA are the microRNAs (miRNAs). These miRNAs are small (~22 nucleotides) single-stranded RNAs that have emerged as important post -transcriptional regulators of gene expression based on limited sequence complementarity.

The aging-associated epigenetic changes influence expression of miRNAs. The role of specific miRNAs in RA has recently been reviewed (121). Here we will review the miRNAs that link changes in gene regulation, aging and RA. Twentyfour miRNAs have been reported to be involved in gene regulation with aging (122). MiRNA's may cause aberrant DNA methylation. For example, the miR-29 gene family, miR-143, miR-148a and miR-152 all target Dnmt3a and Dnmt3b. MiRNAs that are implicated in both gene regulation with aging and in RA are miR-16, miR-124a and miR-125a. The expression level of miR-16 was elevated in PBMC of RA patients with active disease and correlated with the erythrocyte sedimentation rate (ESR), CRP and disease activity score 28 (DAS28) (123). HDAC1, HDAC2 and HDAC3 transcripts are all proven targets of miR-16. It is currently not known if higher miR-16 levels also correspond with lower levels of HDAC transcripts and thus increased histone acetylation in RA patient-derived PBMC. MiR-124a was down regulated in RA synovial fibroblasts (124). MiR-124a targets the 3` UTR of mRNAs encoding MCP-1 and CDK-2. MiR-124a down regulation thus promotes the production of MCP-1 and CDK-2 proteins by RA synovial fibroblasts. Also, miR-124 (among others) is thought to regulate EZH2, a component of the polycomb repressive complex, involved in chromatin remodeling in various cell types.

Pathways most affected by ageing include genes involved in post transcriptional events such as mRNA splicing (125). Hu antigen R (HuR) is one of the splicing control proteins. HuR is an RNA binding protein which stabilizes mRNA, thereby regulating gene expression (126). Increased miR-125a levels correlate inversely with HuR in various tumor cells and miR-125a targets the ELAV gene transcript that encodes the HuR protein levels, thereby regulating HuR expression (126). MiR-125a levels were elevated in plasma from RA patients. These increased plasma levels may reflect the down modulation of HuR expression in PBMC from RA patients (127).

3.4. Loss of protein homeostasis

Exogenous or endogenous stressors can lead to unfolding of proteins. Heat shock proteins (hsps) are stress-induced chaperones that refold these proteins or target them for destruction in autophagosomes. The age-associated decline of hsps is associated with reduced longevity (1).

Interestingly, an increased expression of several hsps in RA patient-derived PBMC (128) as well as in RA synovial tissue and fluid has been reported (129-132). Hsps are upregulated in arthritic joints, likely in response to stress-induced endoplasmic reticulum (ER) hyperreactivity and increased protein turnover. Substantial data support a role for chaperones as autoantigens for T- and B-cells in RA; including detection of hsp-specific autoantibodies (129,130,133-135), presence of autoreactivity-inducing citrulline groups within hsps (136), ability to interact with RA-associated HLA-DRB1*0401 allele (137) and stimulation of T-cell responses (129,130,138,139). In RA patients, hsps serve as targets of the pathological immune response and their levels do not decline as demonstrated in aging.

Damaged, unfolded or incorrectly folded proteins which cannot be re-folded by hsps, are degraded by cellular proteolytic systems such as the ubiquitin-proteasome and lysosomal systems. This process is impaired with age and leads, in tandem with the decrease of chaperone functions, to accumulation and aggregation of erroneous proteins. Together, this results in an age-associated loss of tissue function (1).

Autophagy is a mechanism that involves degradation of cellular components through the actions of lysosomes. The breakdown of cellular components promotes cellular survival during stress by maintaining cellular energy levels. Autophagy is thus an important process relevant to protein homeostasis, energy metabolism and cell death (140). Normal aging generally reduces autophagy (1).

The joint environment imposes ER stress to synovial cells due to a high protein turnover (141-143). Consequently, autophagy is significantly increased in RA synovial fibroblasts. Enhanced autophagy correlated with reduced apoptosis. This led to the assumption that autophagy protects synovial cells from cell death. However, in a recent study using *ex vivo* cultured synovial fibroblasts the authors showed that autophagy may also promote death of RA synovial fibroblasts (144). Both apoptosis-resistance and apoptosis-induction by autophagy represent means of responding to increased levels of stressors within the inflamed RA joints. Modulation of autophagy in synovial fibroblasts in RA joints was shown to depend on TNF-α (144-146).

Similar to the autophagy-lysosomal system, also elements of the ubiquitin-proteasome system have been found altered in RA synovial fibroblasts (144,145,147). A single nucleotide polymorphism (SNP) mapping to the E3 ubiquitin ligase - *cullin1 (CUL1)* gene *locus,* has been associated with RA susceptibility in the Japanese (148) and north Indian populations (149). E3 ubiquitin-protein ligase synoviolin, was increased not only in synovial fibroblasts in the joint (150-152), but also within PBMC and serum of RA patients (153). Synoviolin has been suggested to render synovial cells resistant to apoptosis induced by ER stress (152). Upregulation of synoviolin *in vitro* by fibroblast-like synoviocytes was TNF-α-dependent (151). These data suggest that inflammation-mediated ER stress alters proteostasis

and that altered proteostasis may enhance inflammation, thereby amplifying the local inflammatory response in RA.

Yang *et al* reported that RA naïve T-cells are autophagy-deficient. The authors provide evidence that RA T-cells are in an energy-deprived state and thereby rendered apoptosis-sensitive. Increased apoptosis of naive CD4+ T-cells in RA patients may lead to increased homeostatic proliferation and early senescence of the T-cell pool (154). Thus, metabolic defects may be responsible for loss of proteostasis in RA naïve T cells and may underlie their accelerated senescence.

3.5. Altered Nutrient-Sensing

Pathways involved in nutrient-sensing are thought to have a critical role in aging, as suggested by the overall positive effects of caloric restriction on lifespan. Nutrient-sensing pathways whose dysregulation is consequential for aging include insulin and insulin-like growth factor-1 (IGF-1) signaling (IIS) and the mTOR pathway (1). Intriguingly, IGF-1 deficiency may shorten or extend lifespan, a paradox which remains unresolved at present. Pituitary-derived growth hormone (GH) induces IGF-1 production in the liver, and IGF-1 suppresses GH in a negative feedback loop (155). IGF-1 represents one of two ligands of the IGF family, which also consists of six IGF binding proteins (BP). Availability of IGF-1 for the IGF-1 receptor (IGF-1R) is regulated mostly by IGFBP-3 due to its highest abundance in human serum (156). GH and IGF-1 levels decrease during normal aging (155,157).

Most studies demonstrated decreased levels of IGF-1 (158-161) and increased levels of IGFBP-3 (156,158,162) in serum/plasma of RA patients, which suggests low bioavailability of IGF-1. Acute starvation in RA patients (7 day fasting period) led to a further decline of IGF-1 and reduced measures of inflammation such as ESR, CRP, tender joint count as well as T-cell counts. Interestingly, mitogen activation of CD4+ T-cells after fasting showed increased IL-4 production *in vitro* (163). These data link nutrient-sensing pathways to altered T-cell activation in RA. However, Matsumoto *et al* showed that reduced serum levels of IGF-1 in RA did not correlate with clinical parameters of the disease but were negatively correlated with the age of the patients (158). Moreover, several studies in RA patients failed to detect a change in IGF-1 (164-166) and IGFBP-3 levels (164), and in addition, also observed decreased IGFB-3 levels (160,166). In SF, both IGF-1 and IGFBP-3 levels were shown to be increased (167-169). Thus, the picture is not unequivocal in RA.

Similar contrasting data were obtained for levels of GH (155). Available data show either decreased (166) or increased (159,164) GH levels in the periphery of RA patients. A study in newly diagnosed RA patients demonstrated impaired GH production and, similar to IGF-1, a role of pro-inflammatory cytokines (i.e. IL-1) in the further suppression of GH (170).

Components of the nutrient-sensing machinery, found to be important in aging, such as Akt (protein kinase B), Foxo and mTOR, have been implicated in the local inflammatory process in RA and were

studied mostly in joint-derived FLS (171-176). In RA circulating T-cells, expression of nutrient-sensing proteins (AMPK and mTOR) was not different compared with controls (154).

In summary, alterations of nutrient-sensing pathways in RA seem to be linked to the increased inflammatory status.

3.6. Mitochondrial Dysfunction

Mitochondrial dysfunction is a feature of aging and may be caused by the accumulation of somatic mutations within the mitochondrial genome (mtDNA), and/or by respiratory chain dysfunction, oxidation of mitochondrial proteins and lipids. Relevant to RA pathogenesis are the induction of ROS and mtDNA mutations in synovium. The increase of mtDNA mutations were found only when compared to osteoarthritis (OA) control tissue (177), but not when compared to psoriatic arthritis (PsA) tissue (178). Elevated mtDNA mutation frequency in RA was independent of age. MtDNA mutations positively correlated with macroscopic synovitis, vascularity and with synovial fluid TNF-α and IFN-γ levels. As with other alterations observed within the inflammatory synovial environment, cause-effect relationships are challenging to study. Currently available data suggest involvement of TNF-α, and TNF-α-associated ROS generation in induction of mtDNA mutations (177,178). Mitochondrial dysfunction as a result of compromised metabolism induced by the ATP synthase inhibitor oligomycin, has been shown to promote ROS- and NF-κB-dependent induction of inflammatory markers by normal human synoviocytes (179).

Moreover, cell-free mtDNA has been detected in RA synovial fluid and plasma at levels significantly higher than in healthy subjects (180,181), likely as the result of the increased tissue damage in RA. Study be Collins *et al* demonstrated that cell-free oxidized mtDNA induces development of arthritis upon its intra-articular injection in mice (181).

Chronic inflammation was found to affect the metabolic competence of T-cells in RA. Their capacity to mobilize aerobic glycolysis for ATP generation was significantly reduced (154).

3.7. Cellular senescence

Various cell intrinsic and extrinsic stressors can induce cellular senescence. $P16^{ink4a}$ and p53 play major role in the cellular senescence signaling program (182). Both proteins are central in the regulation of cell cycling and apoptosis. It has been proposed that the p53 and Rb pathways are responsible for the stress-induced growth arrest, while permanent expression of $p16^{ink4a}$ and $p21^{WAF/CIP1}$ maintain the senescence state (183). P53 was overexpressed in synovial tissues from both early and late-stage RA when compared to OA and reactive arthritis, while the cells in normal synovial tissue showed significantly less p53 expression (184). Besides wild-type *p53*, also mutated p53 has been detected in RA synovium (185). Yamanishi *et al* analysed *p53* mutations in RA synovium and demonstrated preferential survival of FLS with the mutated *p53* allele. Interestingly, regions with high p53 expression levels also contained high levels of *IL-6* transcripts (185). This can be explained by the loss of IL-6 suppression dependent on the

wild-type *p53*. Induction of *p53* mutations in synovium has been suggested to represent an event secondary to chronic oxidative stress and the continuous need for DNA repair (184-186).

Upregulation of p16 in synovial fibroblasts leads to permanent growth arrest, a feature associated with the senescent phenotype. The *in vitro* upregulation of p16 in macrophages inhibited IL-6 expression (187). Thus, local induction of p16 expression may have beneficial effects and has been proposed as a possible treatment strategy for RA (188,189).

Interestingly, downregulation of p53 and other proteins from the DDR pathway in naive CD4+ T-cells has been implicated in T-cell senescence. Conversely, p16 and p53 were highly expressed in CD56+CD28- T-cells (13).

Another marker of cellular senescence is SA-β-gal. SA-β-gal was found to be endogenous lysosomal β-gal (190). Although SA-β-gal was detected in OA cartilage, we are not aware of any studies reporting on SA-β-gal expression in RA synovium.

3.8. Stem cell exhaustion

Several studies suggest that alterations at the level of hematopoietic stem cells (HSC) underlie premature aging of the peripheral T-cell pool in RA.

Cells derived from the bone marrow of RA patients showed less effective colony formation potency when compared to HC-derived bone marrow cells (191-193). Further studies confirmed the reduced proliferative capacity of CD34+ HSC from RA patients (194-198). HSC in RA also demonstrated increased apoptosis rate. Concomitantly, the numbers of CD34+ stem cells were found to be decreased in the bone marrow (194-196) and PB in RA (197).

Colmegna *et al* (198) demonstrated a dampened ERK signaling pathway in HSC of RA patients. ERK signaling is involved in the growth, differentiation and prevention of apoptosis (199). More specifically, alteration in RA HSC involved decreased interaction between K-Ras and B-Raf. Ras-induced translocation of Raf to the cellular membrane is necessary for activation of the downstream events of the Raf/MEK/ERK pathway (199). Defects in K-Ras and B-Raf co-localization (and consequently reduced level of activated pERK) have been linked to senescence-associated impairment of the HSC proliferative response in RA.

Impaired proliferative capacity of HSC in RA has also been associated with decreased telomere length. As HSC are the precursors of T-cells, accelerated telomere shortening in RA HSC may underlie the previously reported premature telomere attrition in RA T-cells (5,200). Interestingly, while naive CD4+ T-cells from RA patients were characterized by both telomere erosion and defective telomerase activity, RA HSC demonstrated an increased telomerase activity, suggesting activation of a compensatory pathway (200). Yet, this did not suffice to prevent telomere erosion in HSC of RA patients carrying the RA-cassociated HLA-DRB1*04 alleles and led to the hypothesis of genetically-driven premature immunosenescence in RA (5). Senescence-associated changes within HSC of RA patients were

independent from disease duration or disease activity (197) and did not differ between treated and non-treated patients (194,197). However, Papadaki *et al* showed that increased bone marrow level of TNF-α in RA patients is associated with increased apoptosis and a numerical decrease of CD34+ HSC (194).

3.9. Altered intercellular communication leading to inflammaging

A low-grade inflammation develops with age, while specific immunity wanes. This persistent inflammation has been termed sterile inflammation due to the lack of a defined pathogenic trigger, or inflammaging. Inflammaging has been associated with functional changes of senescent or terminally differentiated cells giving rise to the senescence-associated secretory phenotype (SASP) (3).

Both elderly individuals and patients with RA show increased systemic levels of pro-inflammatory cytokines including, among others, IL-6, IL-8 and TNF-α (201). RA-derived FLS, which undergo replicative senescence induced by serial passage, produce significantly more IL-6, IL-8, vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2) in response to IL-1β, when compared to early-passage FLS (202). Analysis of the molecular mechanisms underlying SASP revealed a critical role of persistent DNA damage response involving increased levels of proteins from the ATM pathway (ATM, NBS1, CHK2) (203,204). In contrast, activated p53 may suppress the senescence-associated cytokine (IL-6, IL-8) secretion, while loss of p53 may amplify the SASP (203-205). Persistent DNA damage has also been suggested as an underlying factor of T-cell replicative senescence in RA. Thus, despite the fact that SASP-inducing ATM and NSB1 were found to be decreased in RA T-cells (77), other proteins involved in the persistent DDR could contribute to SASP in the context of RA. One candidate may involve p53 which was found decreased in RA-derived naive CD4+ T-cells (77).

In conclusion, multiple hallmarks of aging have been identified in early and/or late stage RA patients and are summarized in Table 1.

4. Effects of RA treatment on the aged profile in RA

Biological hallmarks of aging seem to converge in the pre-aged phenotype as seen in RA (Table 1 and Figure 1). Yet, this premature aging may be inflammation-driven. To gain further insight in cause and consequence, we reviewed the effects of anti-inflammatory treatment on the pre-aged phenotype in RA patients. Several researchers addressed the issue of reversibility of premature T-cell aging upon treatment in RA patients. First, *in vitro* studies demonstrated that chronic TNF-α stimulation-induced CD28 loss by T-cells was partly restored by TNF-α inhibition (210,211). Effects of TNF-α blockade on the number of CD28- T-cells in patients, however, are inconclusive. While some studies reported on a reduction of the number of circulating CD4+CD28- T-cells (22,24), others did not observe any change (37). Also, anti-TNF-α treatment did not affect the CMV-specific IFN-γ response of CD4+CD28- T-cells (37). The absolute number of CD4+CD28- T-cells was not different between RA patients receiving abatacept (CTLA-4Ig) or DMARDs (212). Others demonstrated reduction of CD8+CD28- and late-stage CD8

effector memory, but not CD4+CD28- T-cells after 48 weeks of abatacept treatment. The decreases of CD28- cells among both CD4+ and CD8+ T lymphocyte populations after abatacept therapy were correlated with the clinical response as measured by DAS28/CRP (213). Interestingly, the absolute numbers of CD28- T-cells (before treatment) were found to predict the response to abatacept (214).

More importantly, beneficial effects of treatment on premature HSC aging, a putative underlying cause of accelerated T-cell senescence in RA, were reported. The bone marrow niche of RA patients showed decreased apoptosis rates of the CD34+ fraction, increased cell growth potential (assessed by the colony formation assay) and an increased frequency of CD34+ cells after anti-TNF-α treatment (194,195,209). Besides the positive effect on the number of HSC, blocking of TNF-α also resulted in an increase in the levels of recent thymic emigrants, defined as CD31+CD4+ T-cells (209). Parameters of bone marrow function were not different between RA patients who were or were not on concomitant MTX (196), suggesting that TNF-α- but not MTX-mediated suppression of inflammation, is important for rejuvenation of the HSC pool in RA.

Telomere erosion and reduced telomerase activity in CD4+ T-cells were not affected by treatment of RA patients with MTX or prednisone (91,200). Similarly, accelerated telomere erosion in HSC was seen in both treated and not (yet) treated RA patients in cross-sectional analyses only (197). To the best of our knowledge, the influence of anti-TNF-α agents on telomere length and telomerase activity in RA has not yet been addressed. Neutralization of TNF-α *in vitro* was associated with upregulation of telomerase activity in CD8+ T-cells (215).

Effects of DMARD treatment on cellular senescence, markers of DNA damage and epigenetic alterations relevant to RA were studied only in cell line models. *In vitro*-induced senescence in human fibroblast and cancer cell lines demonstrated a glucocorticoid (GC)-mediated reduction of SASP, such as reduced IL-6 and IL-8 expression. GC did not affect persistent DNA damage, the underlying cause of SASP, nor growth arrest or expression of SA-β-gal (216).

Longitudinal clinical studies demonstrated reduction of the markers of oxidative stress, including 8-hydroxydeoxyguanosine upon treatment with MTX or anti-TNF-α agents. These findings suggest that DMARD treatment reduces ROS-induced DNA damage (206,207,217). Also mtDNA mutations frequency, regarded as a marker of mitochondrial dysfunction, was significantly lower in patients with low disease activity (DAS28 <3.2) after TNF-α blocking therapy (178).

Studies investigating effects of anti-TNF-α treatment in RA patients on the activity of HAT and HDAC, regulating the acetylation status of histones, yielded contradictory results. HDAC activity, significantly higher in RA at baseline, did not change upon etanercept treatment (112). Others showed an increase in HAT and a decrease in HDAC activity in a group of RA patients receiving various TNF-α inhibitors and increase of both HAT and HDAC upon RTX treatment (208). Contradictory results were attributed to the use of different biologicals in these studies.

Thus, the combined evidence suggests that inflammation involving TNF-α, drives premature HSC aging, a putative cause of accelerated T-cell senescence in RA. To further substantiate the notion of reversibility of the senescent phenotype by TNF-α blocking agents or DMARDs, longitudinal clinical studies in well-defined patient cohorts before and after treatment are needed to clarify this issue.

Table 1. Hallmarks of aging in early and late RA and their modulation upon anti-TNF-α treatment.

	Aging hallmark	Early RA	Reference	Late RA	Reference	Normalization upon anti-TNF-α treatment	Reference
1.	Genomic instability	yes	(77)	yes	(77,78,81)	yes	(206,207)
2.	Loss of telomeres	yes	(91,93,200)	yes	(90,91,200)	nd	
3.	Gene regulation	nd		yes			
3.1.	Histone modifications	nd		yes	(105,112)	contradictory results	(112,208)
3.2.	DNA methylation	nd		yes	(16,19)	nd	
3.3.	ncRNAs	nd		yes	(123)	nd	
4.	Proteostasis	nd		yes	(128,130, 154)	nd	
5.	Altered nutrient sensing	yes	(170)	yes	(158-161, 164)	nd	
6.	Mitochondrial dysfunction	nd		yes	(178,180)	yes	(178)
7.	Cellular senescence	yes	(184)	yes	(184)	nd	
8.	Stem cell exhaustion	yes	(194,197)	yes	(194,196, 197)	yes	(194,195,209)
9.	Inflammaging*	nd		yes	(14,18,19)	contradictory results	(22,24)

1-4 hallmarks of aging, 5-7 antagonistic hallmarks, 8-9 culprit phenotype.
Hallmarks of aging reflect biological aging but may also be the consequence of inflammation. * The culprit phenotype in RA is represented by pro-inflammatory CD28- T-cells. Pro-inflammatory cytokine production by these cells resembles the SASP. Nd = not determined.

5. Premature aging in RA: cause or consequence?

It has been suggested that premature aging of the immune system may be causal to RA. Telomere shortening of CD4+ T-cells was accelerated in RA patients carrying the RA-associated HLA-DRB1*04 alleles, which led to the hypothesis of genetically-driven premature immunosenescence in RA (5). However, in light of more recent discoveries, we suggest that the causes of premature senescence in RA may be inflammation-driven. New knowledge of how environmental factors interact with susceptibility genes and the immune system in the development of seropositive RA (ACPA+ and/or RF+) has emerged.

ACPA were found to develop more profoundly in individuals carrying the RA-associated HLA-DRB1 shared epitope-containing alleles and can be detected years before symptoms become manifest (218). Presence of ACPA and RF in pre-RA patients was associated with elevated levels of pro-inflammatory cytokines (219,220). ACPA and/or RF may trigger the inflammatory response via an FcγR-mediated mechanism (221,222). Thus, the autoantibody-mediated inflammatory state, that precedes RA development by many years, may be responsible for premature immunosenescence in RA.

6. Conclusions and future perspectives

In order to evaluate the notion that premature immunosenescence in RA is inflammation-driven, we propose to study immunosenescence features in prospective, longitudinal cohort studies. Inclusion of individuals who are at risk for future RA development (not yet receiving DMARD treatment) would allow to assess the association between inflammatory markers and the immunonesenescent phenotype in the preclinical phase of RA, at disease onset and beyond. Cohorts to be used to assess the preclinical alterations putatively implicated in RA pathogenesis are seropositive arthralgia patients (SAP) and patients with early, undifferentiated arthritis (UA). Both UA and SAP have 30% chance of progressing towards classifiable RA (223,224). Furthermore, we propose to study immunesenescence features in late onset seropositive and seronegative RA.

As normal aging is associated with an overall decline of immune function, it will be important to compare healthy age-matched controls, excluding elderly subjects with co-morbidities. Assessment of immunosenescence parameters together with disease activity and pro-inflammatory markers at the preclinical stage and at the switch to clinical synovitis, are expected to provide insight into the mechanisms of their mutual regulation.

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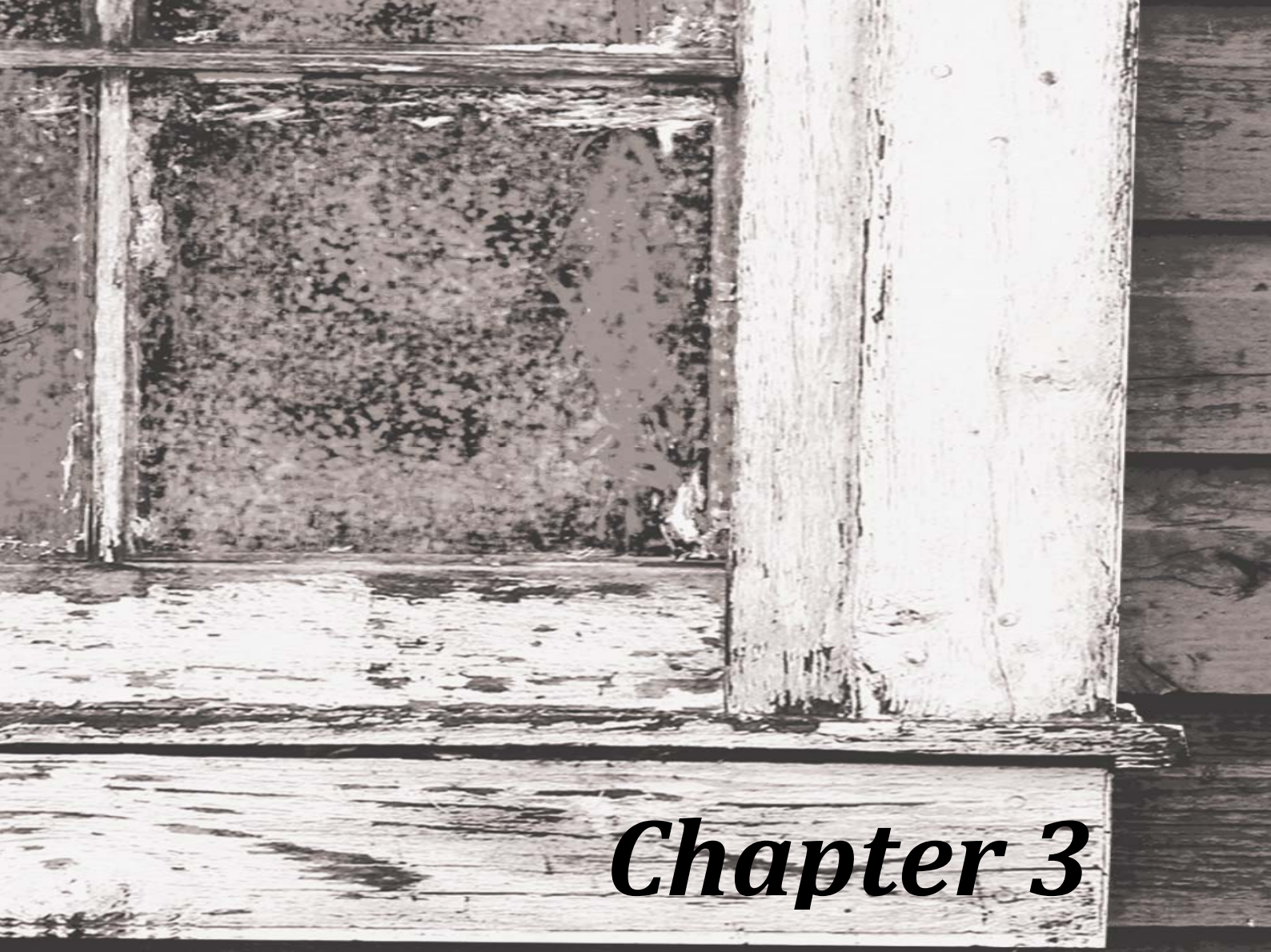

Chapter 3

Serum immune markers discriminate between seropositive and seronegative rheumatoid arthritis and identify high-risk seropositive arthralgia patients

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Submitted

Abstract

Presence of autoantibodies precedes development of seropositive rheumatoid arthritis (SP RA) and seropositive arthralgia patients (SAP) are at risk of developing RA. The aims of the study are to identify additional serum immune markers discriminating between SP and seronegative (SN) RA, and immune markers identifying high-risk SAP.

Sera from SAP (n=27), SP RA (n=22), SN RA (n=11) and healthy controls (n=20) were analyzed using the Human Cytokine 25-Plex Panel. Selected markers were validated in independent cohorts of SP RA (n=36) and SN RA (n=12) patients. Eleven of 27 SAP developed RA within 6.7 months, and their baseline serum markers were compared to 16 non-progressing SAP.

Our data demonstrated that SAP and SP RA patients showed a marked overlap in their systemic immune profiles. Markers increased in SAP and SP RA included IL-2, IL-2R, IL-17, IL-4, IL-15, IL-1β and IL-1RA. In contrast, SN RA showed a distinct immune profile, characterized by increased IL-10 and decreased Eotaxin and Rantes. SAP progressing to RA showed increased IL-5, MIP-1β, IL-1RA and IL-12 levels compared to non-progressing SAP. ROC analysis showed that serum IL-5 most accurately discriminated between the two SAP groups (AUC>0.8), suggesting that baseline IL-5 levels may aid the identification of high-risk SAP.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the synovial membrane. Synovial hyperplasia, neoangiogenesis and invasion of activated innate and acquired immune cells leads to an irreversible destruction of the bone and cartilage of the joint. Aggressive treatment very early in the course of the disease has proven effective in prevention of radiographic progression and tissue damage (1-3). Based on these observations, postponing or even preventing RA development might become feasible by intervening before the onset of all clinical symptoms of RA (4). First-degree relatives of RA patients and seropositive arthralgia patients (SAP) have been suggested to represent groups at high risk of RA development and may thus be eligible for preventive intervention (5).

Seropositivity for autoantibodies such as anti-cyclic citrullinated peptide antibodies (ACPA) and/or rheumatoid factor (RF) is part of the diagnostic criteria for RA (6). Moreover, ACPA and RF levels have a positive predictive value for future RA development and were detected in serum samples up to 18 years before RA diagnosis (7-11). These autoantibodies may have a direct pathogenic effect in RA. In vitro, ACPA-containing immune complexes induced production of pro-inflammatory cytokines via FcγR-dependent triggering of macrophages (12, 13) and presence of IgM RF augmented this process (14). In the pre-clinical stage of RA, emergence of ACPA and RF or increase of ACPA reactivity preceded the elevation of serum cytokine levels (9). It has been suggested that different inflammatory pathways are involved in the development of seronegative (SN RA) and seropositive RA (SP RA). Presence of autoantibodies in early RA has been shown to confer risk of more aggressive, progressive and erosive disease (15-20). SP RA patients have a greater need for disease-modifying anti-rheumatic drugs (DMARDs) or aggressive treatment (16, 17) and a lower chance of achieving drug-free remission (20, 21). Furthermore, presence of ACPA or RF has been associated with the development of comorbidities, such as vasculitis (22), pulmonary diseases (23) and ischemic heart disease (24). Worse clinical outcome suggested increased inflammatory responses in seropositive RA and prompted analysis of the local inflammatory site (25-28). Data on the differences in the systemic inflammatory markers between SP and SN RA is limited (29).

In the present study, we aimed to identify serum immune markers that could discriminate between recently diagnosed SP RA and SN RA patients. Secondly, we aimed to identify baseline serum markers in SAP that could discriminate between SAP who progressed to RA and SAP who did not progress to RA.

Methods

Subjects

In this comparative study we included 22 recently diagnosed SP RA (ACPA+ and/or RF+) patients; 11 recently diagnosed SN RA (ACPA- and RF-) patients; 27 SAP and 20 healthy controls (HC). Serum samples from 36 additional SP RA and 12 additional SN RA were used for validation. Inclusion criteria for the SAP cohort, other than seropositivity, were the presence of arthralgia (tender joint count [TJC] ≥

1) but no diagnosis of arthritis (swollen joint count [SJC] = 0), as assessed by a trained rheumatologist (EB). Early RA patients, fulfilling the 1987 or 2010 American College of Rheumatology (ACR) classification criteria for RA were included at time of diagnosis and these patients did not receive disease modifying anti-rheumatic drugs (DMARDs). Both SAP and RA were treated with non-steroidal anti-inflammatory drugs (NSAIDs) only. Healthy subjects were not recently vaccinated, did not have an infection and did not use immunosupressive drugs at the time of blood withdrawal, as assessed by a health questionnaire. All participants gave their informed consent and the study was approved by the local medical ethics committee (UMC Groningen). All experimental protocols were carried out in accordance with the approved guidelines and were approved by the ethical committee of UMC Groningen. Demographical and clinical characteristics of all study participants are shown in Table 1 and Suppl. Table 1. Eleven of the SAP (41%) progressed to RA (indicated as SAP=>RA) over a median follow-up of 6.7 (range 1.2-32.1) months. The median follow-up time for the non-progressing SAP was 32.0 (range 26.2-33.5) months.

ACPA serum levels were determined by anti-IgG CCP fluorescent enzyme immunoassay using Phadia 250 System (Thermo Fisher Scientific, Uppsala, Sweden) and serum levels ≥ 10 IU/ml were considered as positive. Total RF serum levels were determined by turbidimetry using a modular analyzer (Roche, Mannheim, Germany) and serum levels ≥ 15 IU/ml were considered positive.

Measurement of serum immune markers

Peripheral blood was collected in anticoagulant-free tubes, centrifuged (at 1200 g for 10 min) and serum was stored at -20°C until analysis. Serum immune markers were quantified with the Human Cytokine 25-Plex Panel (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Custom-made Luminex immunoassay (Life Technologies) was used for the detection of IL-1β, IL-15, Eotaxin and Rantes in the validation cohorts. Samples were measured using Luminex 100 System (Luminex, Austin, Tx, USA) and data were analyzed with StarStation software, version 2.3 (AppliedCytometry, Birmingham, UK). The following markers were assessed in the main study: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, IFN-α, IFN-γ, GM-CSF, TNF-α, IL-1 receptor antagonist (IL-1RA), IL-2R, Eotaxin (CCL11), IL-8, IP-10 (CXCL10), MCP-1 (CCL2), MIG (CXCL9), MIP-1α (CCL3), MIP-1β (CCL4) and Rantes (CCL5).

Statistical analysis

Demographical and clinical characteristics were compared with ANOVA or Kruskall-Wallis test for continuous data with normal and non-normal distribution, respectively. Categorical data were analyzed using chi-squared test. Data obtained from 2 groups were compared with Mann-Whitney or Fisher's exact tests. $P < 0.05$ was considered statistically significant. For all analyses, data were log2-transformed in order to approach a Gaussian distribution. Differences between the groups were analyzed with ANOVA

and a Tukey's post-hoc test. In order to adjust for multiple comparisons, results were considered statistically significant when $p \leq 0.002$ (Bonferroni correction). Differences between 2 SAP groups were compared using Mann-Whitney test. Cytokines for the validation study were chosen according to the following criteria: 1) their levels were significantly different between SP RA and SN RA in the main cohort, 2) ≥45% of SP RA and SN RA patients showed expression levels above or below mean ± 2 standard deviations (SD) of the HC values and 3) size of the independent sample cohort required to obtain the desired power (1-sided, sensitivity 90%, confidence intervals 95%) was sufficient. Differences between the groups of SP RA and SN RA from the independent cohorts or from the main cohorts were compared using Mann-Whitney test. Analyses were performed with IBM SPSS Statistics 20 (SPSS, Chicago, IL, USA). Hierarchical clustering analysis was done with Genesis 1.7.6 software (30) using Euclidean distances and average linkage.

Table 1. Baseline demographical and clinical characteristics of the subjects included in the study.

	HC	SAP	SP RA	SN RA
N	20	27	22	11
Age [yrs]; mean (SD)	55.7 (7.5)	50.8 (14.4)	53.4 (12.3)	60.3 (7.5)
Gender; % female (n)	65.0 (13)	66.7 (18)	68.2 (15)	72.7 (8)
ACPA positive; % (n)	NR	92.6 (25)	90.9 (20)	0.0 (0)
RF positive; % (n)	15.0 (3)	88.9 (24)	81.8 (18)	0.0 (0)
CRP [mg/l]; median (range)	NR	5.0 (5.0-29.0)*	12.5 (5.0-75.0)	17.0 (5.0-57.0)
ESR [mm/h]; median (range)	NR	12.0 (2.0-43.0)*	21.0 (2.0-96.0)	45.0 (22.0-88.0)
TJC [n]; median (range)	NR	1.0 (0.0-16.0)*	7.0 (0.0-23.0)	5.0 (0.0-27.0)
SJC [n]; median (range)	NR	0.0 (0.0-0.0)	6.0 (0.0-16.0)	4.0 (0.0-14.0)
DAS28; mean (SD)	NR	NR	4.9 (1.6)	5.0 (1.4)
Erosions; % (n)	NR	NR	13.6 (3)	18.2 (2)

HC: healthy controls; SAP: seropositive arthralgia patients; SP RA: seropositive rheumatoid arthritis patients; SN RA: seronegative rheumatoid arthritis patients; ACPA: anti-cyclic citrullinated proteins antibodies; RF: rheumatoid factor; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; TJC: tender joint count; SJC: swollen joint count; DAS28: disease activity score 28; NR: not reported: * indicates $p<0.05$.

Results

Description of study cohorts

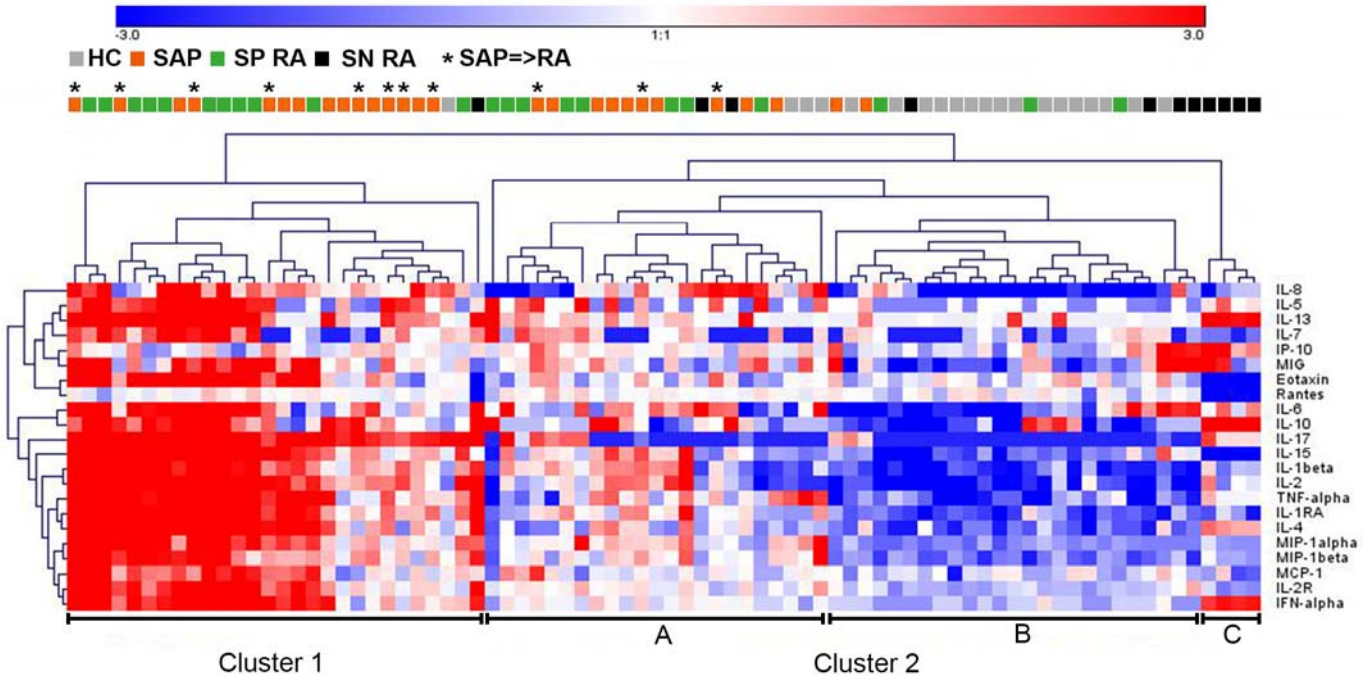
The SAP group was characterized by a significantly lower CRP ($p=0.0055$ and $p=0.0005$, respectively), ESR ($p=0.010$ and $p<0.0001$, respectively) and TJC ($p=0.0002$ and $p=0.0071$, respectively) when compared to SP RA and SN RA patients (Table 1). The percentages of ACPA and RF single- and double-positive patients were similar in the SAP and SP RA patient groups, with the majority being double-positive (ACPA+RF+). Comparison of SP with SN RA patients showed no differences between the baseline characteristics such as age, sex, CRP, ESR, DAS28, SJC, TJC or the rate of joint erosions (Table 1). The independent cohorts of recently diagnosed DMARD-naïve seropositive (n=36) and seronegative (n=12) RA patients included in the validation study did not differ in age, sex, CRP, ESR, TJC, SJC,

DAS28 and erosions rate. Demographical and clinical characteristics of the SP and SN RA patients in the independent cohorts were similar, although age and ESR were lower (p=0.039 and p=0.029, respectively) in the second SN RA cohort (Suppl. Table 1). Comparison of the baseline demographical/clinical characteristics of SAP progressing to RA (SAP=>RA) and SAP showed no differences between the groups. SAP who developed RA tended to be older at the inclusion of the study, compared to SAP not progressing during the follow-up (p=0.058; Suppl. Table 2).

Unsupervised hierarchical analysis of serum immune markers separates SAP and SP RA from SN RA and HC

ANOVA of the 4 study groups: HC, total SAP, SP RA and SN RA revealed significant differences ($p \leq 0.002$) for 22 out of the 25 markers analyzed. IL-12, IFN-γ and GM-CSF were not significantly different between the study groups. Unsupervised hierarchical clustering of the 22 significant markers revealed a separation into 2 clusters (Fig. 1). Fifty-six percent of all SAP and 50% of the SP RA patients form the vast majority of individuals in cluster 1 that is characterized by a higher expression of the 22 markers analyzed. Cluster 2 consisted of three subgroups with 37% of the remaining SAP and 36% of the SP-RA patients grouping together in cluster 2A (intermediate expression levels) and most HC (80%) clustering together in cluster 2B (relative low expression). Cluster 2C was characterized by intermediate expression of the serum immune markers and included 36% of the SN RA patients. The remaining SN RA patients were dispersed among all other clusters. Interestingly, 8/11 SAP who later developed RA (SAP=>RA) were included in cluster 1 (indicated by asterisks).

Figure 1. Unsupervised hierarchical clustering analysis of serum markers from HC, SAP, SP RA and SN RA.



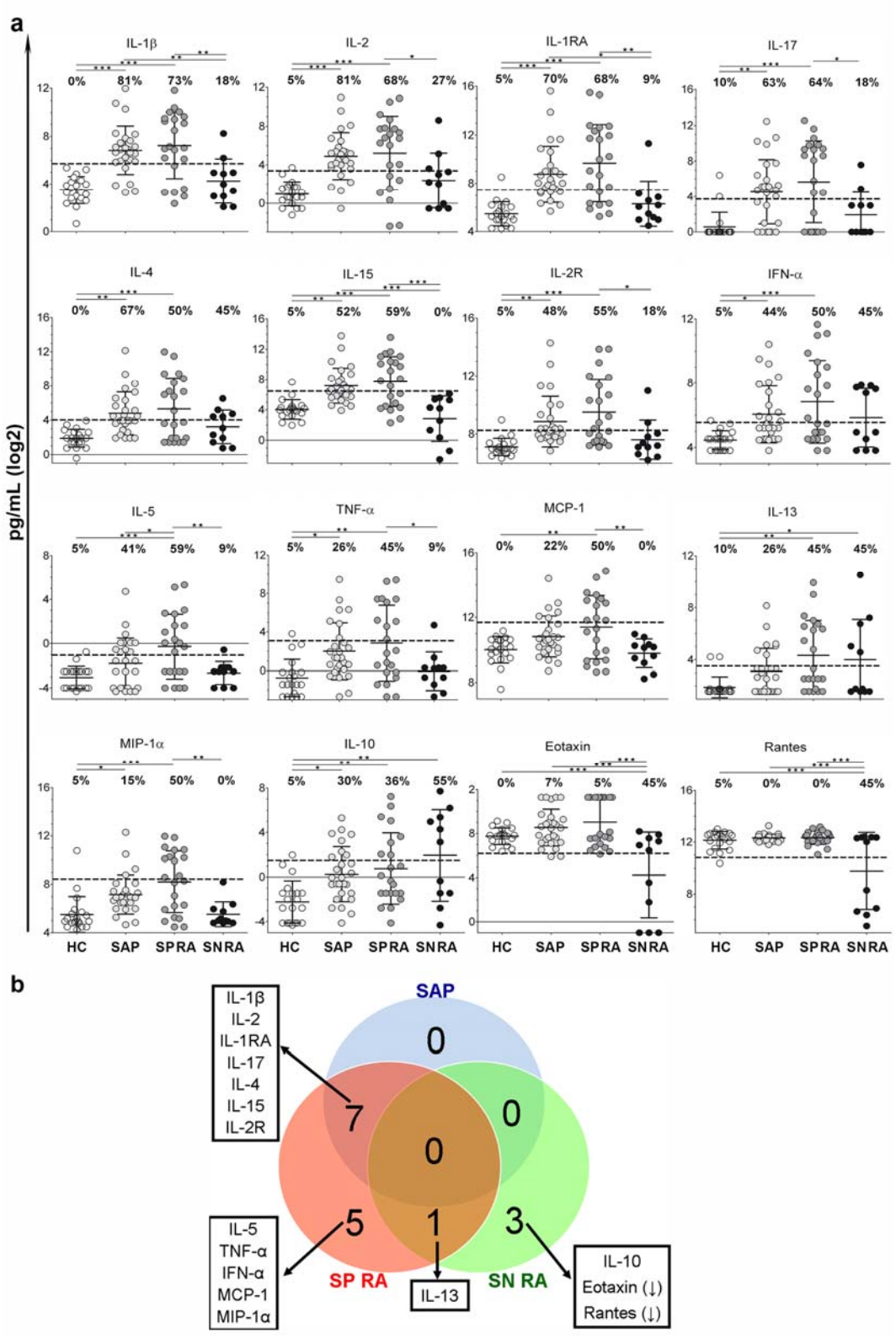
Unsupervised hierarchical clustering (average linkage method, Euclidean distance metric) of the log2-transformed data of 22 serum markers measured in 20 HC, 27 SAP, 22 SP RA and 11 SN RA patients. Asterisks indicate SAP who progressed to RA (SAP=>RA) during follow-up.

In order to identify the most pronounced markers per group, we selected markers that showed an increase/decrease in expression of more than the mean ± 2SD of the HC levels in at least 45% of patients. Sixteen out of 22 markers, showed elevated or decreased levels in ≥45% of patients of at least one group (Fig. 2a). The overlap and differences of the significantly increased or decreased markers in ≥45% of SAP / SP RA / SN RA are visualized in a Venn diagram (Fig. 2b). All markers with increased levels in SAP were also increased in SP RA, i.e. IL-1β (81% and 73%, respectively), IL-2 (81% and 68%), IL-1RA (70% and 68%), IL-17 (63% and 64%), IL-4 (67% and 50%), IL-15 (52% and 59%), and IL-2R (48% and 55%). One cytokine, i.e. IL-13 showed a similar upregulation in SP RA and SN RA (45% and 45%), but not in SAP (26%). The markers that showed a pronounced upregulation in SP RA but not in the other groups were IL-5 (59%), MCP-1 (50%), MIP-1α (50%), IFN-α (50%) and TNF-α (45%). IL-10 serum levels were increased above the cut-off only in SN RA patients (55%). In the SAP and SP RA groups the IL-10 levels were also increased, but the percentages of patients with an increase above the cut-off value were only 30% and 36%, respectively. Next to the pronounced increase of IL-10, SN RA patients had decreased levels of Eotaxin and Rantes in 45% of patients. These decreases were not observed in the other groups (Fig. 2).

Validation of serum immune markers in independent SP RA and SN RA cohorts

To verify the differences between early SP RA and SN RA patients, we repeated the measurement of a selected set of serum markers (n=4) in independent SP RA and SN RA cohorts (Table 2). Power analyses indicated that the validation cohort size was sufficient to detect significant differences for IL-1β, IL-15, Eotaxin and Rantes. Significantly higher levels of IL-1β, IL-15 and Eotaxin were detected in SP RA compared to SN RA ($p=0.011$, $p=0.029$ and $p=0.025$, respectively, Table 2) confirming the differences found in the initial cohort. The decreased levels of Rantes in SN RA compared to SP RA could not be confirmed. Thus, 3 of 4 cytokines discriminating between SP RA and SN RA were validated in the independent cohorts.

Figure 2. Serum markers in patient groups compared to HC.



Graphs depict expression levels of log2-transformed values in HC, SAP, SP RA and SN RA. The dotted line indicates the threshold of mean ± 2SD of HC values. Horizontal lines represent mean and whiskers represent SD. Percentages above the data sets indicate the frequency of subjects showing expression values above/below the threshold. Differences between the groups were calculated using ANOVA and post-hoc Tukey's test with the significance indicated as *** for $p \leq 0.0005$; ** for $p \leq 0.01$ and * for $p < 0.05$. B) Venn diagram showing differences and overlap in serum markers who were 1) statistically different between patient groups when compared to HC, and 2) were increased/decreased above/below mean ± 2SD of HC values in ≥45% SAP, SP RA or SN RA.

Table 2. Results of the validation study in independent SP RA and SN RA cohorts.

Validation cohorts			
	SP RA	**SN RA**	**p-value**
N	36	12	-
IL-1β [pg/mL(log2)]; median (IQ range)	4.74 (4.46-5.44)	4.35 (4.23-4.60)	**0.011**
IL-15 [pg/mL(log2)]; median (IQ range)	4.82 (4.53-5.19)	4.45 (4.24-4.79)	**0.029**
Eotaxin [pg/mL(log2)]; median (IQ range)	6.51 (6.14-6.83)	5.15 (3.07-7.06)	**0.025**
Rantes [pg/mL(log2)]; median (IQ range)	12.98 (12.55-13.42)	13.06 (12.82-13.20)	0.96
Main cohorts			
	SP RA	**SN RA**	**p-value**
N	22	11	-
IL-1β [pg/mL (log2)]; median (IQ range)	7.10 (5.05-9.54)	3.98 (3.00-4.96)	**0.004**
IL-15 [pg/mL (log2)]; median (IQ range)	7.95 (4.42-10.55)	4.18 (0.35-5.38)	**0.002**
Eotaxin [pg/mL (log2)]; median (IQ range)	8.03 (6.99-11.19)	6.48 (-1.00-7.57)	**0.003**
Rantes [pg/mL (log2)]; median (IQ range)	12.45 (12.06-12.69)	12.02 (6.65-12.37)	**0.011**

SP RA: seropositive rheumatoid arthritis patients; SN RA: seronegative rheumatoid arthritis patients. SP RA and SN RA cohorts from the validation or the main study were compared with Mann-Whitney test. $P<0.05$ was considered statistically significant.

Baseline levels of serum markers identifying high-risk SAP

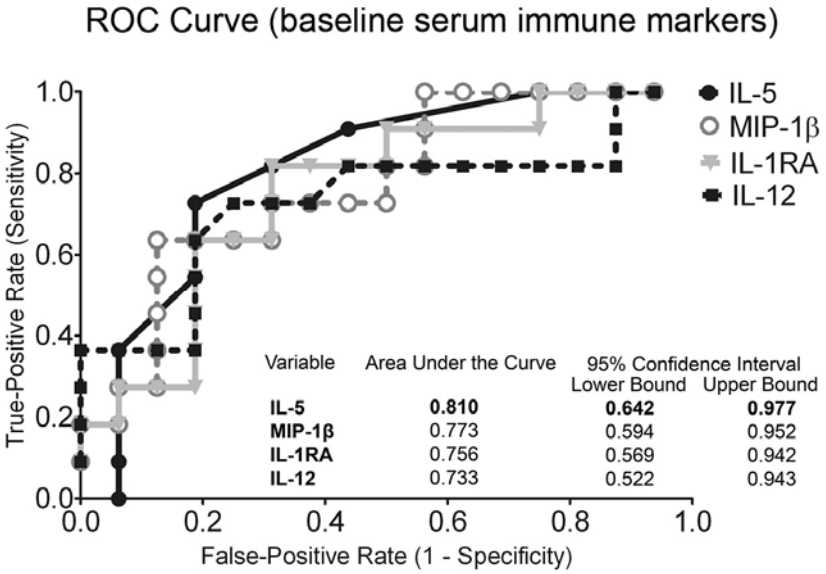
We investigated whether the baseline serum markers differed between SAP who progressed to RA (SAP=>RA, median time to progression 6.7 months) and SAP who did not progress to RA during the follow-up period (median follow-up 32 months). Eleven of the 27 (41%) SAP progressed to SP RA (Fig. 1, Suppl. Table 2). SAP=>RA were characterized by higher baseline levels of IL-5, MIP-1β, IL-RA and IL-12, compared to SAP who did not progress to RA (Table 3). Receiver operating characteristic (ROC) analysis was used to determine if baseline levels of any of these 4 immune markers may discriminate between SAP who progress to RA from SAP who do not. A good discriminatory ability (Area Under the Curve, [AUC] >0.8) was obtained for IL-5 (Fig. 3). Our data suggest that baseline IL-5 levels may help to identify SAP at risk for future RA development.

Table 3. Baseline levels of serum markers in SAP who progressed to RA and SAP not progressing.

Immune marker	SAP not progressing (n=16)	SAP=>RA (n=11)	p-value
	[pg/mL(log2)]; median (IQ range)		
IL-5	-4.0 (-4.24- -1.58)	-0.54 (-1.58-0.07)	**0.007**
MIP-1β	7.50 (6.22-7.99)	8.24 (7.55-9.54)	**0.019**
IL-1RA	7.61 (6.75-8.59)	9.02 (8.03-11.64)	**0.028**
IL-12	7.95 (7.73-8.10)	8.15 (8.00-9.41)	**0.046**
IL-2	4.34 (2.57-5.38)	5.22 (4.01-8.10)	0.109
IL-6	2.23 (0.42-4.13)	3.03 (2.45-4.59)	0.109
IL-1β	6.66 (4.33-7.14)	7.04 (6.05-10.30)	0.132
IL-7	3.32 (2.00-5.08)	5.06 (2.00-6.92)	0.167
IL-2R	7.96 (7.77-8.90)	8.37 (8.14-10.00)	0.175
Eotaxin	7.81 (7.11-9.21)	8.86 (7.63-9.74)	0.199
IL-13	1.68 (1.58-3.32)	3.32 (2.55-4.71)	0.204
MIP-1α	6.84 (6.07-7.40)	6.99 (6.48-9.29)	0.267
IFN-α	5.20 (4.52-7.07)	5.63 (5.20-8.04)	0.275
IL-17	3.70 (0.00-7.02)	4.78 (4.45-6.42)	0.286
TNF-α	0.98 (-0.55-4.23)	1.32 (0.55-3.38)	0.311
IL-15	6.49 (5.66-7.80)	7.03 (5.73-10.38)	0.336
IFN-γ	-1.74 (-2.00- -1.00)	-1.00 (-2.00- -0.61)	0.388
IL-10	-0.33 (-2.58-2.89)	0.70 (-0.65-1.96)	0.401
GM-CSF	4.60 (2.24-5.93)	5.00 (4.26-5.79)	0.412
MCP-1	10.42 (10.05-10.94)	10.69 (9.84-12.07)	0.430
IP-10	3.98 (3.80-4.52)	4.34 (3.78-5.49)	0.570
Rantes	12.32 (12.15-12.52)	12.33 (12.13-12.42)	0.604
IL-8	8.62 (8.06-9.94)	9.43 (8.16-11.20)	0.639
MIG	3.77 (2.75-4.69)	3.12 (3.08-5.20)	0.902
IL-4	4.21 (3.12-5.52)	3.94 (2.59-6.56)	1.000

SAP: seropositive arthralgia patients. SAP groups were compared using Mann-Whitney test. P<0.05 was considered statistically significant.

Figure 3. Receiver operating characteristic curves (ROC) for selected baseline serum markers in SAP=>RA and SAP not progressing.



ROC analysis and area under the curve of ROC curves was performed for 4 immune markers whose baseline levels show significant difference (p<0.05; Mann-Whitney test) between SAP=> RA and SAP not progressing (as demonstrated in Table 3).

Discussion

The aims of the present study were to compare serum immune markers for their ability to discriminate between early SP and SN RA; and to identify serum immune markers that may predict progression to RA in SAP.

It has been suggested that RA does not begin at the level of the joint but is preceded by systemic inflammation (9). This is supported by several studies that demonstrated systemic elevation of various inflammatory factors in the pre-RA stage (10, 11, 31). Analysis of the markers of systemic inflammation in SAP, who are at risk of RA development (5), has not yet been performed. Analysis of the local inflammation in SAP showed either weak (32, 33) or lack of (34) signs of subclinical synovitis in SAP.

One of the conclusions of the present study is that the increase in markers of systemic inflammation is also a feature of SAP, and that the SAP immune profile is highly similar to the profile seen in SP RA patients. The marked overlap of serum markers in SAP and SP RA reflects a common inflammatory background between both conditions with increased levels of IL-1β, IL-1RA, associated with general inflammation; increased levels of T-cell activation markers (IL-2, IL-2R, IL-4) and increased levels of markers associated with Th17-specific activation (IL-17, IL-1β, IL-15). IL-1β levels were elevated in most SAP and SP RA patients. This was mirrored by elevations in IL-1RA. The concomitant increase of IL-1β and IL-1RA indicates activation of both pro- and anti-inflammatory pathways. Despite the observed increase of IL-2, known to promote Th1 and Treg cells and inhibit Th17 differentiation (35, 36), no alterations of Th1-type cytokines (IFN-γ, IL-12) or the Treg-associated IL-10 were observed in SAP and SP RA. In contrast, IL-17 was significantly increased in these two SP groups. Thus, our results undermine the notion of RA as a Th1-mediated disease and support a role of Th17 cells in the early stages of SP RA pathogenesis, as previously suggested by others (25, 26). Moreover, increased levels of IL-1β and IL-15 in the periphery of SAP and SP RA may contribute to maintaining pathogenic Th17 responses, as they have been demonstrated to promote Th17 differentiation (37) and trigger IL-17 expression (38), respectively.

The second conclusion from the present study is that, in contrast to SAP and SP RA, SN RA patients showed a unique immune marker profile, characterized by a decrease of Eotaxin and Rantes and an increase of IL-10. Eotaxin, a chemoattractant for eosinophils, basophils, mast cells and Th2 cells (39), is induced by IL-1β, IL-4, IL-13 or TNF-α (40, 41), and inhibited by IL-10 (40). In view of these data, the decline of Eotaxin in SN RA compared to HC, as confirmed by the validation study, could be the result of elevated IL-10 levels. However, the causes and implications of the Eotaxin decrease in SN RA are currently unclear and require further study.

Despite the differences in pro- and anti-inflammatory markers between SP RA and SN RA, clinical features of these groups at baseline were similar. Most available studies showed that, in line with our cohorts, all or most of the baseline demographical and clinical characteristics were similar between ACPA+ and ACPA- RA patients (16, 17, 25, 27, 28). However, significantly increased CRP, ESR and

DAS28 levels, and increased radiographic damage in ACPA+ patients have also been reported (16, 18, 19, 27). It has been suggested that differences in the pathogenesis and prognosis between SP RA and SN RA are the consequence of different pathological events at the inflammatory site. However, most studies reported similar levels of inflammatory markers in the joints of SP RA and SN RA, with significantly increased levels being observed only for CCL20, IL-10, IL-1β and IL-17 (25, 26) in ACPA+ RA. Increased lymphocytic infiltration, expression of T-cell markers and lymphocyte chemoattractant in the synovium of ACPA+ compared to ACPA- RA patients has been reported (27). These differences in synovial infiltration between ACPA+ and ACPA- RA patients, however, were not confirmed by three other studies (25, 26, 28). Also, the numbers of B-cells, plasma cells in the synovium (25, 27, 28) or B-cells in synovial fluid and blood (42) were found to be similar between seropositive and seronegative RA. Thus, there is no consensus on synovial markers discriminating between SP and SN RA. Our study is the first to describe specific differences in serum immune markers in SP RA and SN RA. Deane et al, reported that the percentage of pre-diagnosis samples positive for cytokines was lower in patients who later developed SN RA as compared to the percentage of cytokine positive samples in patients who later developed SP RA (29). ACPA/RF-containing immune complexes can trigger cytokine production via FcγR-crosslinking, as demonstrated in vitro (12-14). We hypothesize that this mechanism is responsible for the observed more pronounced expression of serum markers in SP RA compared to SN RA. The qualitative differences between SP RA and SN RA indicate the importance of stratifying RA patients according to the autoantibody status in studies investigating pathological pathways involved in RA and in clinical trials.

The third conclusion of this study is that baseline levels of IL-5 may aid in identifying high risk SAP. The percentage of SAP who developed RA in our cohort was similar to that reported by others (32, 43, 44). The role of IL-5 in RA; a Th2-specific cytokine primarily involved in regulation of eosinophil functions in the tissue (45), is ill-defined. IL-5 was not present in the synovium and rheumatoid nodules of RA patients (46, 47). Implications of the increase of systemic IL-5 levels in SAP=>RA, a serum marker that was also found elevated in 59% of SP RA, would require further studies. So far, the identification of high risk SAP relied mostly on demographic (i.e. presence of the first-degree relative with RA, alcohol non-use) and clinical variables (i.e. duration of the morning stiffness ≥ 1 hour, symptoms and VAS pain ≥ 50) (43). Recently, the combination of a type I IFN signature with a B cell^{low} signature was found to predict RA development in SAP (44, 48). Our data suggest that measurement of serum IL-5 may add to current prediction models.

Supplementary Table 1. Baseline demographic and clinical characteristics of the subjects included in the validation study.

	SP RA validation cohort	P-value compared to SP RA main cohort	SN RA validation cohort	P-value compared to SN RA main cohort
N	36	–	12	–
Age [yrs]; mean (SD)	54.0 (9.9)	ns	49.8 (14.2)	0.039
Gender; % female (n)	63.9 (23)	ns	66.7 (8)	ns
ACPA positive; % (n)	86.1 (31)	ns	0.0 (0)	–
RF positive; % (n)	100.0 (36)	0.046	0.0 (0)	–
CRP [mg/l]; median (range)	8.5 (5.0-104.0)	ns	5.0 (5.0-63.0)	ns
ESR [mm/h]; median (range)	28.0 (3.0-83.0)	ns	24.5 (3.0-48.0)	0.029
TJC [n]; median (range)	5.0 (0.0-28.0)	ns	8.5 (0.0-24.0)	ns
SJC [n]; median (range)	5.0 (0.0-28.0)	ns	9.0 (0.0-25.0)	ns
DAS28; mean (SD)	4.6 (1.6)	ns	5.8 (2.3)	ns
Erosions; % (n)	27.8 (10)	ns	16.7 (2)	ns

SP RA: seropositive rheumatoid arthritis; SN RA: seronegative rheumatoid arthritis; ACPA: anti-cyclic citrullinated proteins antibodies; RF: rheumatoid factor; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; TJC: tender joint count; SJC: swollen joint count; DAS28: disease activity score 28; ns = not statistically significant. Groups were compared using Mann-Whitney test. $P<0.05$ was considered statistically significant.

Supplementary Table 2. Baseline demographic and clinical characteristics of SAP not progressing and SAP=>RA.

	SAP not progressing	SAP=>RA
N	16	11
Age [yrs]; mean (SD)	47.3 (12.1)	55.8 (16.4)
Gender; % female (n)	75.0 (12)	54.5 (6)
ACPA positive; % (n)	87.5 (14)	90.9 (10)
RF positive; % (n)	87.5 (14)	90.9 (10)
CRP [mg/l]; median (range)	5.0 (5.0-29.0)	5.0 (5.0-19.0)
ESR [mm/h]; median (range)	14.0 (2.0-32.0)	11.0 (5.0-43.0)
TJC [n]; median (range)	1.0 (0.0-11.0)	2.0 (0.0-16.0)

SAP: seropositive arthralgia patients; ACPA: anti-cyclic citrullinated proteins antibodies; RF: rheumatoid factor; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; TJC: tender joint count.

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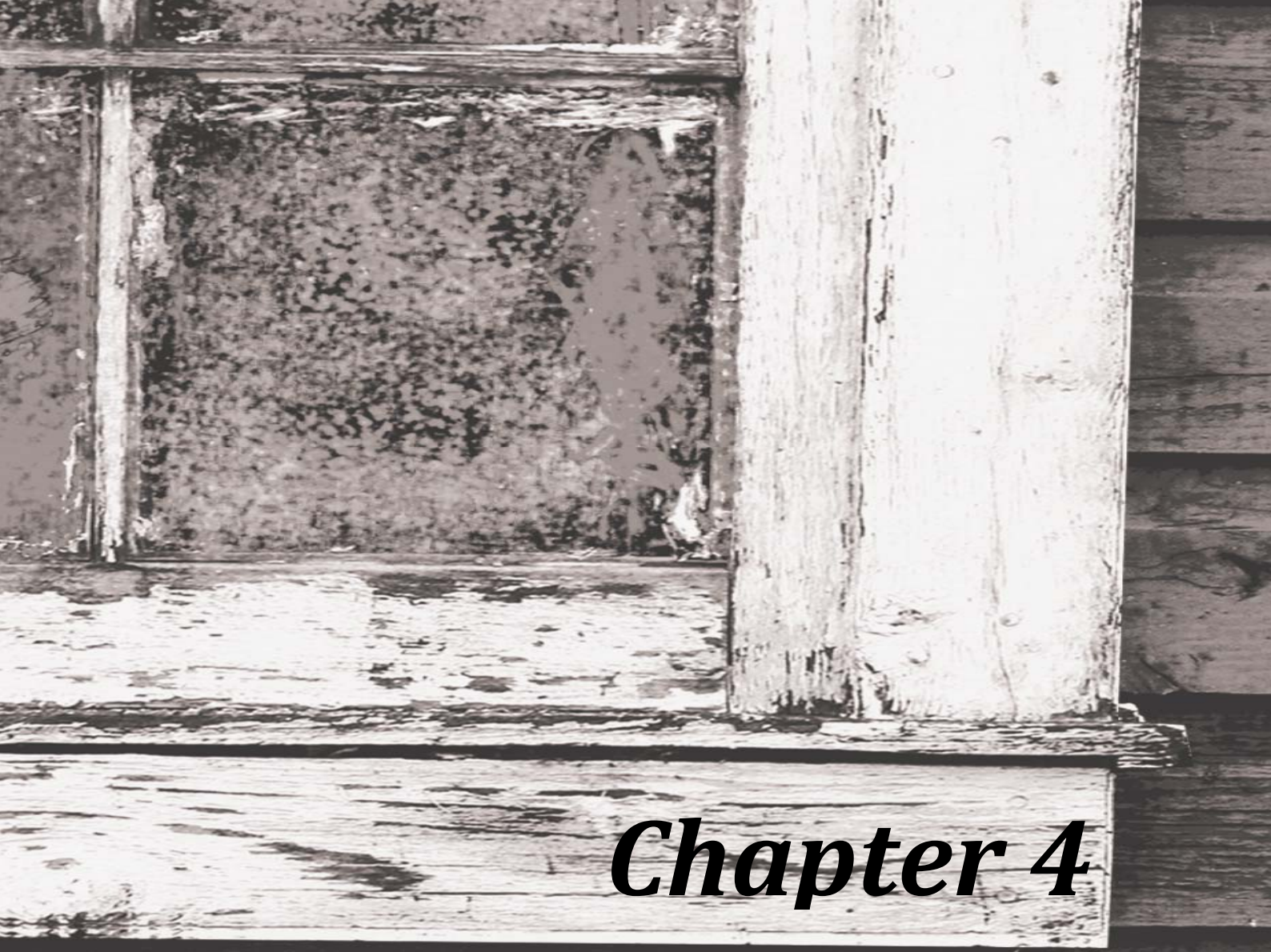

Chapter 4

Altered NK-cell subsets in seropositive arthralgia and early rheumatoid arthritis patients are associated with autoantibody status

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Submitted

Abstract

Objective

The role of NK-cells in the immunopathogenesis of RA is unclear. Therefore, numerical and functional alterations of $CD56^{dim}$ and $CD56^{bright}$ NK-cells in the early stages of RA development were studied.

Methods

Whole blood samples from newly diagnosed, treatment-naïve, seropositive and seronegative RA patients (SP RA, n=45 and SN RA, n=12), seropositive arthralgia patients (SAP, n=30) and healthy controls (n=41) were assessed for numbers and frequencies of T-cells, B-cells and NK-cells. Seropositive status was defined as anti-cyclic citrullinated peptide antibodies [ACPA] and/or rheumatoid factor [RF]) positive. Peripheral blood mononuclear cells were used for further analysis of NK-cell phenotype and function.

Results

Total NK-cell numbers were decreased in SP RA and SAP but not in SN RA. Also, NK-cells from SP RA showed a decreased potency for IFN-γ production. A selective decrease of $CD56^{dim}$, but not $CD56^{bright}$ NK-cells in SP RA and SAP was observed. This prompted investigation of CD16 (FcγRIIIa) triggering in NK-cell apoptosis and cytokine expression. In vitro, CD16 triggering induced apoptosis of $CD56^{dim}$, but not $CD56^{bright}$ NK-cells from healthy controls which was augmented by adding IL-2. Also, CD16 triggering in the presence of IL-2 stimulated IFN-γ expression by $CD56^{dim}$ NK-cells.

Conclusions

The decline of $CD56^{dim}$ NK-cells in SAP and SP RA and the in vitro apoptosis of $CD56^{dim}$ NK-cells upon CD16 triggering, suggest a functional role of IgG-containing autoantibody (ACPA and/or RF)-immune complexes in this process. Moreover, CD16-triggered cytokine production by $CD56^{dim}$ NK-cells may contribute to the pro-inflammatory state as seen in SAP and SP RA.

Introduction

Rheumatoid arthritis (RA) is a chronic autoinflammatory disease. RA is manifested by inflammation of the synovial membrane mediated by joint-infiltrating immune cells. Increased expression of numerous cytokines and cytokine receptors has been observed early in the disease pathogenesis (1-4). This poses a challenge for understanding the primary events in immune dysregulation involved in RA development. Current data support a role of IL-17 in the early phases of RA (3, 5). Despite the originally postulated pathogenicity of IFN-γ, several reports demonstrated its protective role in the development of collagen-induced arthritis (CIA), a mouse model of RA (6-8). The exact protective mechanism of IFN-γ in RA is currently not fully known (9). NK-cells are primary IFN-γ producers (9) by which they connect to the adaptive immune response and favor Th1 cell polarization in the course of an inflammatory response (10-12). NK-cell depletion was found to accelerate CIA onset which was associated with an impaired IFN-γ-dependent regulation of the Th17 response (7). Also, NK-cells contribute to immune tolerance through killing autoreactive T-cells and B-cells (13, 14).

NK-cells are divided into 2 major subsets based on the expression of CD56 (neural cell adhesion molecule, NCAM) (15). $CD56^{dim}$ NK-cells, which constitute ~90% of peripheral blood NK-cells are characterized by a potent cytotoxic capacity associated with increased perforin, granzyme and cytolytic granule expression (16). This suggests a primary role for $CD56^{dim}$ NK-cells in killing of autoreactive cells. In addition, $CD56^{dim}$ cells are more effective in antibody-dependent cellular cytoxicity (ADCC) when compared to the $CD56^{bright}$ subset, as a result of higher surface expression of FcγRIIIa (CD16). $CD56^{bright}$ NK-cells are the minor subset (~10%) within the circulating NK-cell pool. However, in secondary lymphoid organs (e.g. lymph nodes (17, 18)) and at several inflammatory sites (e.g. synovial fluid (19), psoriatic plaques (20)) $CD56^{bright}$ NK-cells have been shown to outnumber $CD56^{dim}$ cells. $CD56^{bright}$ NK-cells may also have an immunoregulatory role due to an increased ability (compared to $CD56^{dim}$ subset) to produce pro- and anti-inflammatory cytokines (15, 16, 21-25).

Given their potentially initiating capacity in skewing and regulating the immune response, we aimed to investigate the role of NK-cells in early stages of RA development. We studied newly diagnosed, treatment-naïve RA patients and seropositive arthralgia patients (anti-cyclic citrullinated peptide antibodies [ACPA] and/or rheumatoid factor [RF]) positive, without synovitis [SAP]). Previous studies show that 35% of SAP develop RA after approximately 1 year of follow-up (26, 27). In the present study we focused on $CD56^{dim}$ and $CD56^{bright}$ NK-cell subsets, specifically in relation to their differential function in the immune response.

Material and methods

Patients

Thirty SAP were defined based on seropositivity for RF (serum levels ≥ 15 IU/mL) and/or ACPA (serum levels ≥ 10 IU/mL), arthralgia in at least one joint and lack of arthritis. Also, 45 early RA patients

seropositive for ACPA and/or RF, 12 early seronegative RA patients (ACPA- and RF-) and 41 healthy controls (HC) were included in the study (Table 1). All RA patients, fulfilling 1987 or 2010 American College of Rheumatology (ACR) classification criteria for RA, were included in the study at the time of diagnosis, before start of treatment with DMARDs. SAP and RA patients received non-steroidal anti-inflammatory drugs (NSAIDs) only. HC were included only if, at the time of blood withdrawal, they had no infections, no recent vaccination and did not use immunosupressive drugs. All participants gave their written informed consent and the study was approved by the local medical ethics committee (University Medical Center Groningen, The Netherlands).

Table 1. Demographical and clinical characteristics of the subjects included in the study.

	HC	SAP	SP RA	SN RA
N	41	30	45	12
Age [yrs]; mean (SD)	50.3 (11.7)	50.7 (14.4)	57.4 (14.0)	64.3 (8.4)
Gender; % female (n)	68.3 (28)	70.0 (21)	80.0 (36)	75.0 (9)
CRP [mg/l]; median (range)	nd	5.0 (5.0-29.0)	12.0 (5.0-108.0)	16.5 (5.0-57.0)
ESR [mm/h]; median (range)	nd	12.0 (2.0-69.0)	24.0 (2.0-96.0)	38.5 (11.0-88.0)
DAS28; mean (SD)	na	na	4.8 (1.4)	5.0 (1.4)
ACPA positive; % (n)	nd	93.3 (28)	91.1 (41)	0.0 (0)
RF positive; % (n)	nd	83.3 (25)	91.1 (41)	0.0 (0)
Erosions; % (n)	na	na	22.2 (10)	16.7 (2)

HC = healthy controls; SAP = seropositive arthralgia patients; SP RA = seropositive rheumatoid arthritis; SN RA = seronegative rheumatoid arthritis; ACPA = anti-cyclic citrullinated proteins antibodies; RF = rheumatoid factor; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; DAS28 = disease activity score 28; na = not applicable; nd = not defined

Analysis of circulating leukocyte populations

Whole blood was analyzed using BD MultiTest™ TruCount method with reagents detecting CD45, CD3, CD4, CD8, CD19, CD16/CD56, according to the manufacturer's instructions (BD Biosciences, Breda, The Netherlands). Flow cytometry was performed on FACS Canto II and analysis was performed using FACS Canto Clinical Software (BD Biosciences).

Analysis of NK-cell phenotype and function

Heparin blood was used to isolate mononuclear cells (PBMC) by Lymphoprep™ (Axis-Shield, Oslo, Norway) density gradient centrifugation and PBMC were processed for cryopreservation. PBMC from all subjects were thawed at the same time and stained with the following antibodies: CD3 eFluor605NC, CD57 eF450 (eBioscience, Vienna, Austria), CD56 FITC, CD16 Alexa Fluor700, CD94 APC, NKG2D PE-Cy7 (BioLegend, San Diego, CA, USA), NKG2A PerCP (R&D Systems, Abingdon, UK), KIR2DL4 (Exbio Praha, Vestec, Czech Republic).

To assess NK-cell IFN-γ expression, thawed PBMC were resuspended in RPMI-1640 containing 10% FBS and 0.6% gentamicin (Life Technologies, Bleiswijk, The Netherlands) at a concentration of 10^6 cells/100 µL. Cells were incubated with phorbol myristate acetate (PMA) at a final concentration of 50 ng/mL, calcium ionophore at a final concentration of 1.6 µg/mL (both from Sigma-Aldrich, Zwijndrecht, The Netherlands) and BD GolgiPlug™ (BD Biosciences) diluted 1:1000. After 4h at 37°C, PBMC were stained with the following antibodies: CD3 eFluor605NC, CD56 PE (eBioscience), CD69 PE-Cy (BioLegend), FasL (CD178) Alexa Fluor 647 (AbD Serotec, Puchheim, Germany). Cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and stained with anti-IFN-γ Brilliant Violet 421 antibody (BioLegend).

To assess NK-cell degranulation potency, analysis of CD107a was performed. Briefly, thawed PBMC were resuspended in RPMI-1640 with 10% FBS and 0.6% gentamycin at a concentration of 10^6 cells/100 µL. Cells were stimulated with PMA at the same concentrations as mentioned above in the presence of 0.5 µg of anti-CD107a Brilliant Violet 421 (BioLegend) antibody. After 1h, BD GolgiPlug™ (diluted 1:1000) and BD GolgiStop™ (diluted 1:1000, both from BD Biosciences) were added and the stimulation was continued for another 5 hours. After washing, PBMC were stained with antibodies: CD3 eFluor605NC and CD56 PE (eBioscience). PBMC were analyzed using LSR II flow cytometer (BD Biosciences). Data analysis was performed with Kaluza® analysis software (Beckman Coulter).

NK-cell isolation and in vitro culture

For NK-cell isolation and culture, blood from healthy volunteers (n=6) was used and PBMC were isolated by Lymphoprep™ (Axis-Shield) density gradient centrifugation. PBMC were resuspended in PBS with 2 mM EDTA, 0.5% BSA and incubated with antibodies: CD3 eF450, CD56 PE and CD19 PE-Cy7 (eBioscience). NK-cell subsets: CD3-CD19-$CD56^{dim}$ and CD3-CD19-$CD56^{bright}$ were isolated by fluorescence-activated cell sorting using MoFlo Astrios sorter (Beckman Coulter, Woerden, The Netherlands, Fig.3A). Sorted $CD56^{dim}$ and $CD56^{bright}$ NK-cells were resuspended in RPMI with 0.6% gentamicin and 5% FBS (Lonza) to a concentration of $5x10^5$ cells/mL and incubated for 16h at 37°C in 96-well flat-bottom polystyrene plates (Thermo Fisher Scientific). Culture conditions included 1000 U/mL human recombinant IL-2 (PeproTech, London, UK), heat-aggregated rabbit IgG (RAGG; Sigma-Aldrich) at a final concentration of 100 µg/mL, anti-CD16 antibody (clone 3G8; BioLegend) at a final concentration of 1 µg/mL, both IL-2 and RAGG, both IL-2 and anti-CD16 or medium alone. RAGG was prepared as described (28).

Analysis of $CD56^{dim}$ and $CD56^{bright}$ NK-cell apoptosis in vitro

After 16h incubation, cell suspensions of sorted $CD56^{dim}$ and $CD56^{bright}$ NK-cells were centrifuged, supernatant was collected and stored at -20°C until analysis. Cell pellets were washed with PBS and resuspended with 3,3 -dihexyloxacarbocyanine iodide ($DiOC_6(3)$; Life Technologies) at a final

concentration of 40 nM. After 15 min incubation at 37°C, cells were washed with PBS and analyzed immediately using LSR II flow cytometer (BD Biosciences). Data analysis was performed with Kaluza® analysis software (Beckman Coulter).

Detection of cytokines in supernatants from cultured CD56^{dim} and CD56^{bright} NK-cells

Levels of IFN-γ, TNF-α, IL-12, IL-4, IL-5 and IL-6 in the culture supernatants were quantified using Human Th1/Th2 Essential 6-plex Luminex assay (eBioscience) according to the manufacturer's instructions. Data analysis was performed using StarStation software, version 2.3 (Applied Cytometry, Birmingham, UK).

Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics 20 (SPSS, Chicago, IL, USA). Normally distributed data were analyzed using an unpaired t test. Skewed data were analyzed using the Mann-Whitney 2-tailed test. Paired samples were analyzed with the Wilcoxon signed-rank test. $P<0.05$ was considered statistically significant.

Results

Seropositive patients (SAP and RA), but not seronegative RA patients, are characterized by a decline of circulating NK-cells

We aimed to identify peripheral immune alterations putatively involved in the early stages of RA pathogenesis and therefore compared the composition of the circulating lymphocyte pool (CD4+ T-cells, CD8+ T-cells, B-cells and NK-cells) between patients (SAP, early SP RA and early SN RA) and HC (Fig.1, Suppl. Fig.1). The number of NK-cells was significantly decreased in SAP and SP RA compared to HC (median 0.21 in SAP and 0.19 in SP RA vs. 0.30 x10^6 NK-cells/mL in HC). A similar decrease was observed for the NK-cell percentage within the total CD45+ pool (median 10.15% in SAP and 10.90% in SP RA vs. 13.64% in HC). In contrast, the absolute number and the proportion of NK-cells were not altered in early SN RA patients (median 0.33 x10^6 NK-cells/mL and 19.31%, respectively) (Fig.1A). No significant alterations in the number of other circulating lymphocyte subsets were observed between patients and HC (Suppl.Fig.1).

NK-cells can be divided into 2 phenotypically and functionally distinct subsets based on CD56 expression (Fig.1B). The absolute number of CD56^{dim} NK-cells was decreased in both SAP (median 0.19 x10^6 cells/mL) and SP RA (median 0.17 x10^6 cells/mL) compared to both HC (median 0.27 x10^6 cells/mL) and SN RA (median 0.29 x10^6 cells/mL). The frequencies of CD56^{dim} NK-cells (within total CD3- cells), however, were not altered (Fig.1C). In contrast, the absolute numbers of CD56^{bright} NK-cells were not different in SAP (median 0.014 x10^6 cells/mL) or SP RA (median 0.018 x10^6 cells/mL) when compared to

HC (median 0.022 x10^6 cells/mL). SN RA patients showed a specific increase of $CD56^{bright}$ NK-cells in both their absolute number (median 0.040 x10^6 cells/mL) and frequency (median 4.44% of $CD56^{bright}$ cells within CD3-) when compared to HC (2.01%), SAP (1.99%) and SP RA patients (2.55%) (Fig.1 D).

The mean age of SN RA patients was higher than the mean age of the HC (Table 1). To exclude the possibility that the observed outcome is confounded by the age difference, a multiple linear regression analysis was performed. After adjusting for age, the previously observed differences in the absolute number and the frequency of $CD56^{bright}$ NK-cells between HC and SN RA patients ($p<0.005$ and $p=0.028$, respectively) remained statistically significant (analysis not shown).

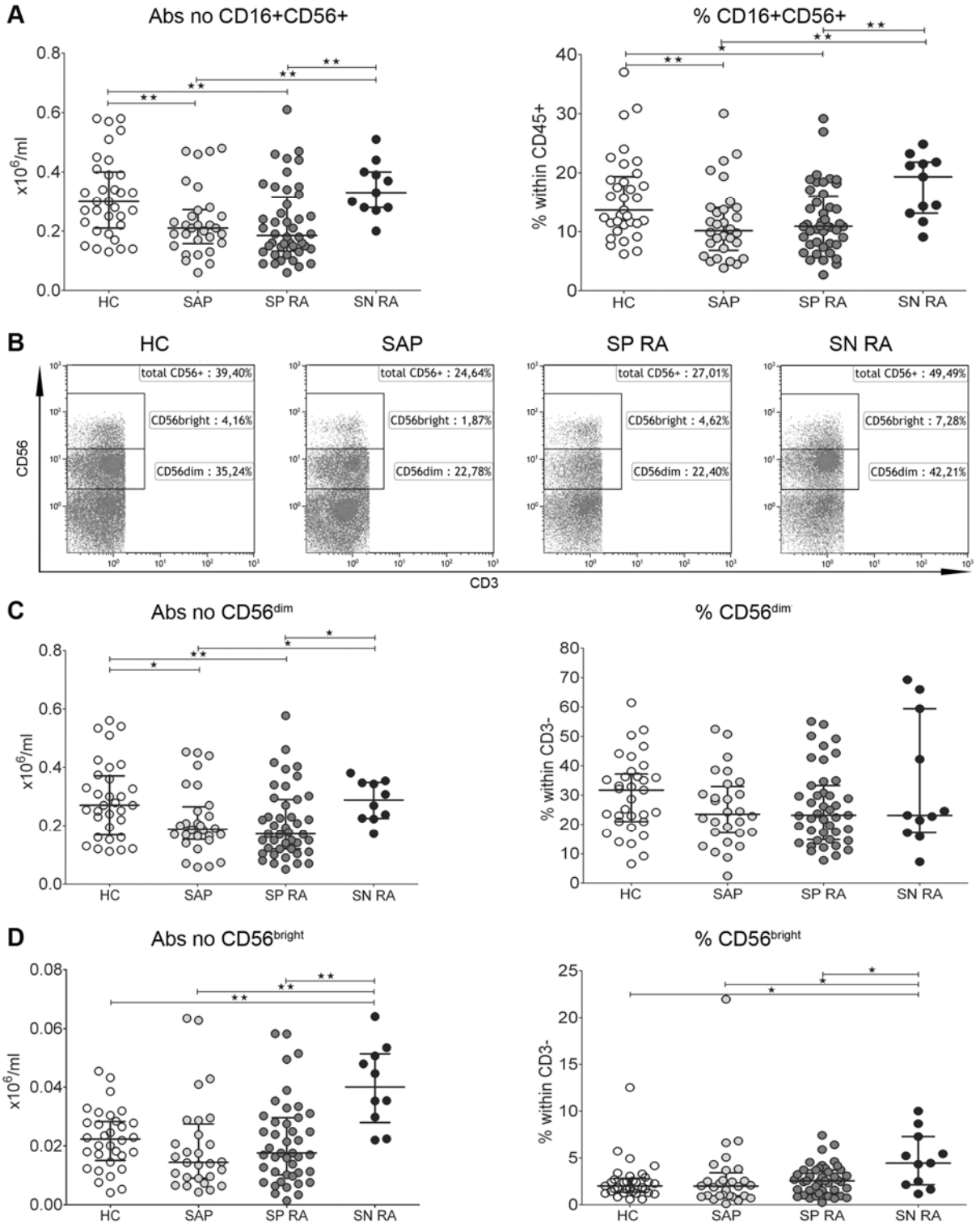
Next, we assessed whether the decline of NK-cell numbers was associated with markers of general inflammation (CRP, ESR) or disease-specific characteristics (DAS28, ACPA or RF level). We found a weak negative correlation between RF level and the absolute number of total NK-cells ($p=0.034$ and $r=-0.23$; data not shown).

NK-cells from seropositive RA patients showed decreased IFN-γ expression

As we found the NK-cell pool altered in SAP and early SP RA patients, we next investigated their functionality by analyzing intracellular expression of CD107a and IFN-γ following PMA/Ca ionophore stimulation in vitro. Spontaneous expression of these markers did not differ between the groups (data not shown). NK-cells from recently diagnosed SP RA patients showed a decreased potency to produce IFN-γ compared to HC (median 51.4% vs 58.9% IFN-γ+ cells within CD3-CD56+ NK-cells in SP RA and HC, respectively; Fig.2A). This was not observed for NK-cells from SAP. Thus, the lesser capacity for IFN-γ production by NK-cells from SP RA does not seem to be caused by the decline of $CD56^{dim}$ NK-cells. No statistically significant differences in CD107a expression were observed between the studied groups (Fig.2B).

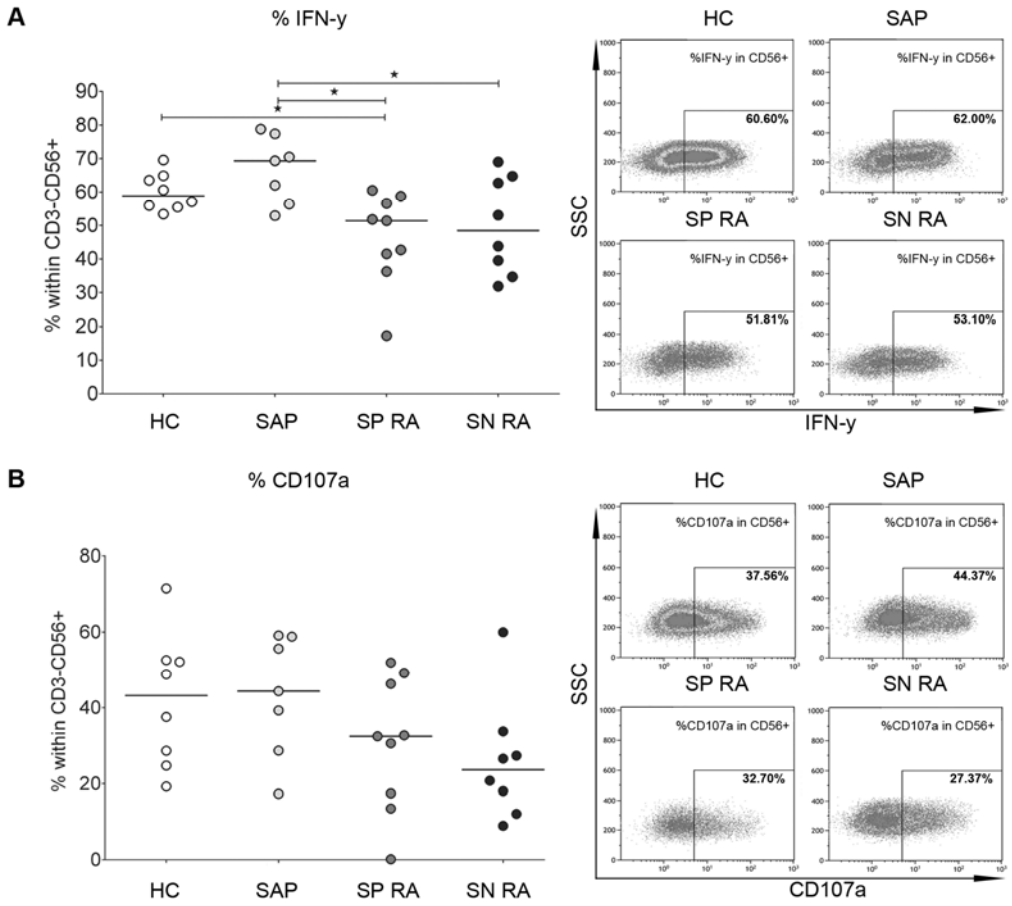
NK-cell function was also analyzed indirectly by assessing the surface expression of receptors with an activating (NKG2D, CD57), inhibitory (CD94/NKG2A) or activating/inhibitory role (KIR2DL4). CD56+ NK-cells from SP RA showed significantly higher expression of NKG2D when compared to HC (median 74.1% vs. 63.1% NKG2D+ cells within CD3-CD56+ NK-cells in SP RA and HC, respectively). This alteration was observed within both $CD56^{dim}$ and $CD56^{bright}$ NK-cell subsets. No other differences in the expression of NKG2D or CD57, CD94/NKG2A and KIR2DL4 between the studied groups were observed (Suppl.Fig.2). These data suggest an altered functionality of the peripheral NK pool in SP RA, but not in SAP.

Figure 1. Decrease of NK-cells in SAP and newly diagnosed, seropositive RA.



(A) Absolute number and frequency of CD56+CD16+ NK-cells in the blood of HC (n=33), SAP (n=30), early SP RA (n=44) and SN RA (n=11). (B) Representative dot plots from HC, SAP, SP RA and SN RA patient showing the gating strategy to analyze $CD56^{dim}$ and $CD56^{bright}$ cells within the CD3-CD56+ NK-cell population. The absolute number and the frequency of (C) $CD56^{dim}$ and (D) $CD56^{bright}$ NK-cells was assessed using PBMC from HC (n=32), SAP (n=28), early SP RA (n=43) and early SN RA patients (n=10). Statistical significance: * $p < 0.05$; ** $p < 0.001$.

Figure 2. NK-cells from early SP RA show decreased intracellular expression of IFN-γ.



PBMC from HC (n=8), SAP (n=7), early SP RA (n=9) and SN RA (n=8) were stimulated for 4h with PMA (50 ng/mL) and calcium ionophore (1.6 μg/mL). After gating for CD3-CD56+ cells, the frequency of (A) IFN-γ+ and (B) CD107a+ cells was assessed. Representative dot plots of 1 subject from each group are shown. Statistical significance: * $p < 0.05$.

CD56^{dim} and CD56^{bright} NK-cell subsets: different susceptibility to FcγRIIIa- induced apoptosis

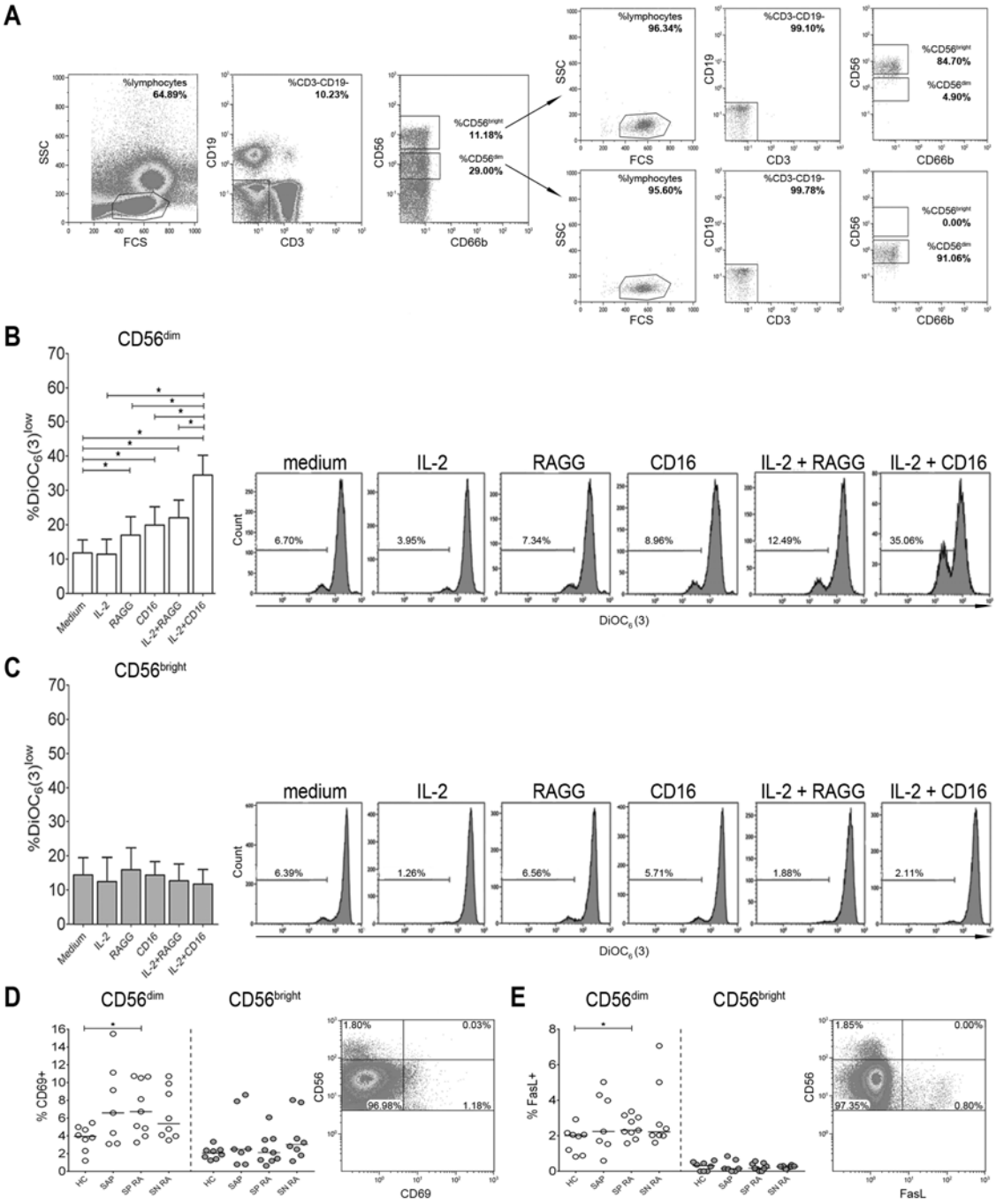
We next assessed if the reduced NK-cell numbers in SAP and SP RA might be explained by immune complex-mediated induction of NK-cell apoptosis via FcγRIIIa (CD16)-triggering. CD56^{dim} and CD56^{bright} NK-cells were isolated from the blood of healthy volunteers and incubated with rabbit aggregated IgG (RAGG) or agonistic anti-CD16 antibody in the presence or absence of recombinant IL-2. RAGG has been demonstrated to bind FcγR on NK-cells and to mirror RF immune complexes (29). Apoptosis was assessed by $DiOC_6(3)$ uptake analysis. RAGG and anti-CD16 alone enhanced apoptosis of cultured CD56^{dim} NK-cells (median 12.68% and 14.14% DiOC6(3)^{low} cells, respectively, Fig.3A,B) compared to the control (medium alone, 8.53% DiOC6(3)^{low} cells). Apoptosis of CD56^{dim} NK-cells was further increased in the presence of IL-2 (35.06% DiOC6(3)^{low} cells). IL-2 alone had no effect on the number of apoptotic cells in vitro. In contrast to CD56^{dim} NK-cells, RAGG or anti-CD16 did not enhance apoptosis of CD56^{bright} NK-cells (Fig.3A,C).

Previously, culture of NK-cells with anti-CD16 antibody was found to induce apoptosis, cytotoxicity, proliferation, TNF-α expression and phenotypic changes, such as upregulation of CD69 and FasL (30, 31). Thus, we assessed whether NK-cells isolated from seropositive patients (SAP and SP RA) showed

increased expression of CD69 and FasL, indicating functional FcγRIII-triggering in vivo. Both CD69 and FasL were expressed at a higher level by CD56^{dim} (median 3.9% and 2.0%, respectively) than CD56^{bright} (median 2.1% and 0.3%, respectively) cells in healthy controls. In SP RA, the percentages of CD69+ and FasL+ cells within CD56^{dim} NK-cells were significantly increased (6.7%, p=0.027 and 2.3%, p=0.036; respectively) when compared with HC (Fig.3D,E). A similar pattern was observed for CD56^{dim} NK-cells in SAP but this did not reach statistical significance.

In conclusion, our in vitro data show that CD56^{dim} and not CD56^{bright} NK-cells undergo apoptosis upon FcγRIII-triggering in vitro, with the number of apoptotic cells increasing further upon addition of IL-2.

Figure 3. CD56^{dim} but not CD56^{bright} NK-cells undergo apoptosis upon FcγRIIIa-triggering.



(A) Gating strategy used in sorting for CD56^{dim} and CD56^{bright} NK-cells and re-analysis of the purity of sorted NK-cell subsets are shown (median 85% and 96%, respectively). CD56^{dim} and CD56^{bright} NK-cells were cultured with

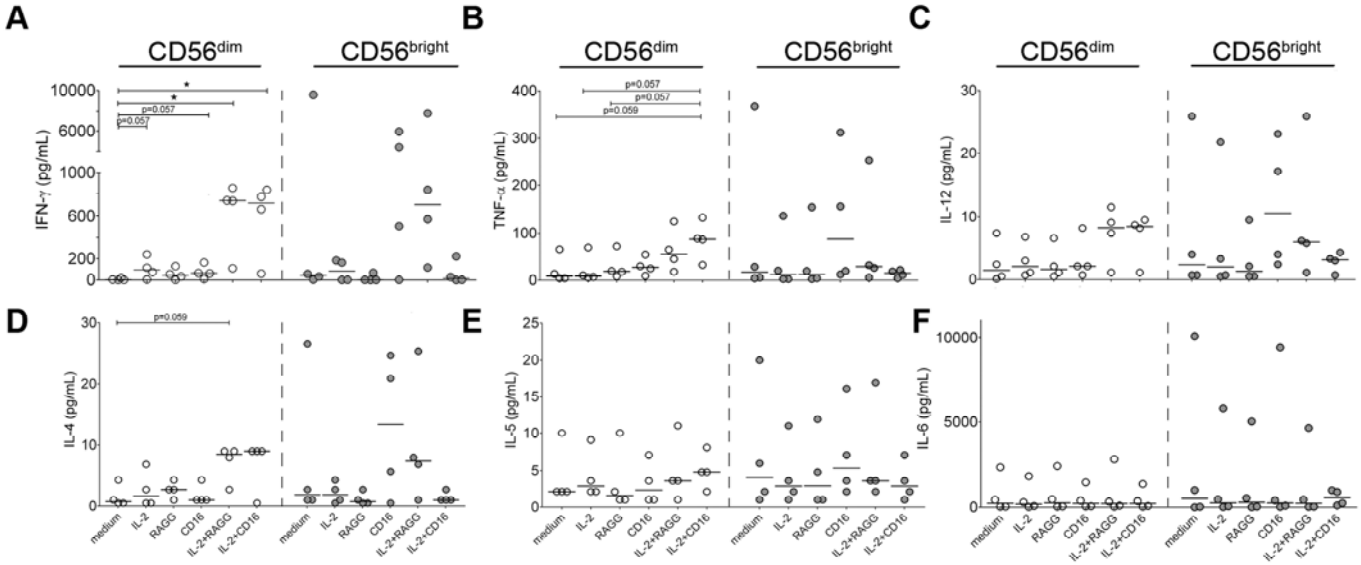
IL-2, RAGG, anti-CD16, IL-2 and RAGG, IL-2 and anti-CD16 or medium alone. Frequency of $DiOC_6(3)^{low}$ cells within (B) $CD56^{dim}$ and (C) $CD56^{bright}$ events. Representative histograms from 1 HC are shown. (D) CD69 and (E) FasL expression within non-stimulated $CD56^{dim}$ and $CD56^{bright}$ NK-cells from HC (n=8), SAP (n=7), SP RA (n=9) and SN RA (n=8). Representative dot plots from 1 HC are shown. Statistical significance: * $p < 0.05$.

$CD56^{dim}$ and $CD56^{bright}$ NK-cell subsets: different propensity to produce IFN-γ following FcγRIIIa triggering

We next assessed the effect of CD16 triggering on cytokine production by NK-cells from healthy donors. Sorted $CD56^{dim}$ and $CD56^{bright}$ NK-cells were cultured with medium alone, IL-2, RAGG, anti-CD16, IL-2+RAGG or IL-2+anti-CD16 and supernatant was used to assess the production of IFN-γ, TNF-α, IL-12, IL-4, IL-5 and IL-6. IL-2+RAGG or IL-2+anti-CD16 stimulation induced significant upregulation of IFN-γ production, specifically in $CD56^{dim}$ NK-cells. The median fold increases in IFN-γ levels when compared to cultures with medium alone were 161 and 126 times, respectively. A trend (p=0.057) toward increased TNF-α production by $CD56^{dim}$ NK-cells cultured in the presence of IL-2 and anti-CD16 was also observed. No statistically significant increase in cytokine expression was observed for $CD56^{bright}$ NK-cell cultures (Fig. 4).

Taken together, the observed decline of $CD56^{dim}$ NK-cells in SP RA and SAP may be mechanistically explained by RF/ACPA immune complex-mediated induction of CD16-dependent apoptosis. Moreover, CD16 triggered cytokine production by $CD56^{dim}$ NK-cells may contribute to the early pro-inflammatory state as seen in SAP and SP RA.

Figure 4. $CD56^{dim}$ but not $CD56^{bright}$ NK-cells produce IFN-γ upon FcγRIIIa-triggering.



$CD56^{dim}$ and $CD56^{bright}$ NK-cells were sorted and cultured as described in Fig.3. After 16h, supernatants from various culture conditions of $CD56^{dim}$ and $CD56^{bright}$ NK-cells (from 4 HC used for Fig.3) were collected. Levels of (A) IFN-γ, (B) TNF-α, (C) IL-12, (D) IL-4, (E) IL-5 and (F) IL-6 were assessed using 6-plex cytokine assay. Statistical significance: * $p < 0.05$.

Discussion

We show a profound decline of NK-cells in recently diagnosed RA and in seropositive arthralgia patients, representing subjects at risk of progressing towards RA (26, 27). By stratifying our RA cohort according to autoantibody status, we found the NK-cell decrease associated with seropositive, but not seronegative RA. Moreover, the decline in NK-cells may be explained by a selective decrease of $CD56^{dim}$ NK-cells as a result of apoptosis induction via FcγR triggering by IgG-containing immune complexes. In line with published data (29-34), we observed the occurrence of FcγR- dependent (anti-CD16-induced) apoptosis of NK-cells, augmented by IL-2, in vitro. We demonstrated differential susceptibility of $CD56^{dim}$ and $CD56^{bright}$ NK-cell subsets to FcγR- induced cell death.

The robust decline of NK-cells in SAP and in recently diagnosed SP RA suggests that this alteration may contribute to disease development rather than represent the consequence of long-term inflammation. Most of the published data describe similarly decreased NK-cell numbers in later stages of RA (35-37). The use of NSAIDs was found to have no effect on peripheral NK-cell numbers (38). Thus, NSAIDs are unlikely causal to the NK-cell decline in SAP and SP RA.

Previously, NK-cell depletion was found to accelerate the onset and augment the severity of CIA. Following the decline of NK-cells, the decrease in systemic IFN-γ levels led to an expansion of Th17 cells directly involved in CIA induction. Furthermore, the NK-cell decrease was associated with plasma cell development and increased systemic levels of IgG autoantibodies (7). This, together with the here described decline of NK-cells in SP RA, but not in SN RA, suggests a protective role for NK-cells in RA development.

Despite similarly reduced NK-cell numbers in SAP and early SP RA, the decline of IFN-γ expression was observed in the latter group only. Thus, as shown for CIA (7), the progression of pre-RA to overt disease may be associated with a reduction of NK-cells as well as their functional impairment. This would confirm the beneficial role of IFN-γ in arthritis pathology as shown in CIA (6-8) and RA (39).

The decline of peripheral NK-cell numbers in seropositive, but not in seronegative patients, as well as the previously reported induction of NK-cell apoptosis by FcγR- triggering (29, 30, 33) suggested a role of RA-related IgG- containing autoantibodies in this process. The majority of ACPAs are IgG (40) and can be bound by IgM RF (41). A study by Boros et al showed that IgM from RA sera was reactive with FcγRIII (42). Furthermore, about half of RA patients have RF in a form of small IgG complexes (43), which are efficiently bound by FcγRIIIa (44).

We confirmed induction of NK-cell apoptosis by agonistic anti-CD16 antibody and rabbit aggregated IgG in vitro, a process that was enhanced by addition of IL-2 (29, 30, 33). We observed a higher sensitivity of sorted $CD56^{dim}$ NK-cells to FcγR crosslinking-induced apoptosis, which is likely the result of the higher expression of CD16 compared to $CD56^{bright}$ cells (15). Differential susceptibility of $CD56^{dim}$ and $CD56^{bright}$ NK-cells to FcγR- dependent apoptosis corresponds to the decline of circulating $CD56^{dim}$ but not $CD56^{bright}$ NK-cells in the seropositive patients in vivo. It is unlikely that the decline of $CD56^{dim}$ NK-

cells in the periphery is a result of a preferential recruitment of this population to the joints as SF-derived NK-cells were mainly of the $CD56^{bright}$ phenotype (data not shown, (19)).

Our hypothesis of autoantibody-mediated stimulation of NK-cells in vivo is supported by the increased expression of CD69 and FasL by non-stimulated $CD56^{dim}$ NK-cells in SP RA patients. Upregulation of both these markers has been demonstrated upon in vitro culture of isolated NK-cells or NK-cell clones in the presence of anti-CD16 antibody (30, 31). FasL, expressed upon FcγRIII-triggering, has been shown to facilitate NK-cell apoptosis in an autocrine manner (30), thereby contributing to enhanced cell death.

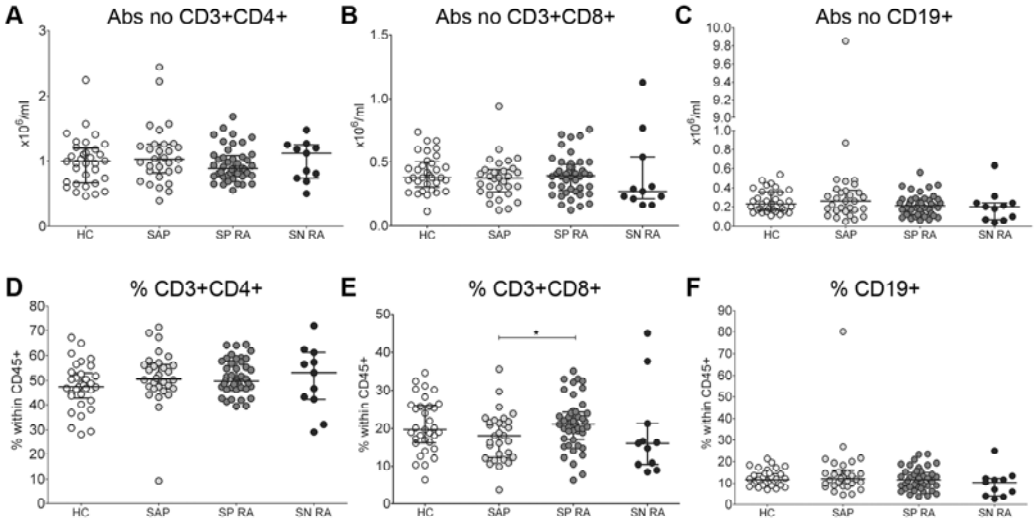
We observed an increased number of $CD56^{bright}$ NK-cells in seronegative RA. Prior data suggests that the expansion of $CD56^{bright}$ NK-cells is more specific for autoimmune diseases such as systemic lupus erythematosus (SLE) (45) or multiple sclerosis (46) than RA and was associated with increased levels of type I interferons. As suggested by Meyer, in contrast to "classical" ACPA- and RF- positive RA with a central role of TNF-α, seronegative RA shares more similarities with SLE (47).

As shown previously (31, 32), CD16 triggering of $CD56^{dim}$ NK-cells induced production of IFN-γ and TNF-α, cytokines implicated in RA pathogenesis. This process was augmented by the addition of IL-2. We observed that the subset of $CD56^{dim}$ NK-cells may be primarily responsible for the enhanced cytokine expression, although this feature has previously been attributed to $CD56^{bright}$ NK-cells (15, 16, 21-25). Increased IFN-γ and TNF-α expression by $CD56^{bright}$ compared to $CD56^{dim}$ was previously seen following stimulation with combinations of monocyte-derived cytokines (21-23), PMA/ionomycin (16, 22) or whole bacterial pathogen (25). Involvement of CD16 in the modulation of $CD56^{dim}$ NK-cell cytokine expression has also been demonstrated (48-50). Thus, pro-inflammatory cytokine production cannot be exclusively attributed to $CD56^{bright}$ NK-cells. Depending on the available stimulus, both NK-cell subsets can produce cytokines.

We propose that the interaction between NK-cells and RA-specific IgG-containing immune complexes is an early event in disease development. This is in line with the notion that the emergence of ACPA and RF autoantibodies occurs years before RA onset (3). FcγRIIIa- triggering of $CD56^{dim}$ NK-cells by autoantibody-immune complexes could result in activation and cytokine expression. Persistent FcγRIIIa-triggering, however, in a pro-inflammatory environment may lead to loss of function and a higher sensitivity to apoptosis of the $CD56^{dim}$ NK-cell subset. The latter process is accelerated by IL-2 which, similar to ACPA and RF, was found increased in the periphery at the pre-RA stage (3). Activation of $CD56^{dim}$ NK-cells may thus contribute to the pro-inflammatory state as seen in SAP and SP RA. Moreover, the decline of $CD56^{dim}$ NK-cells may allow for uncontrolled expansion of autoimmune cells contributing to RA development.

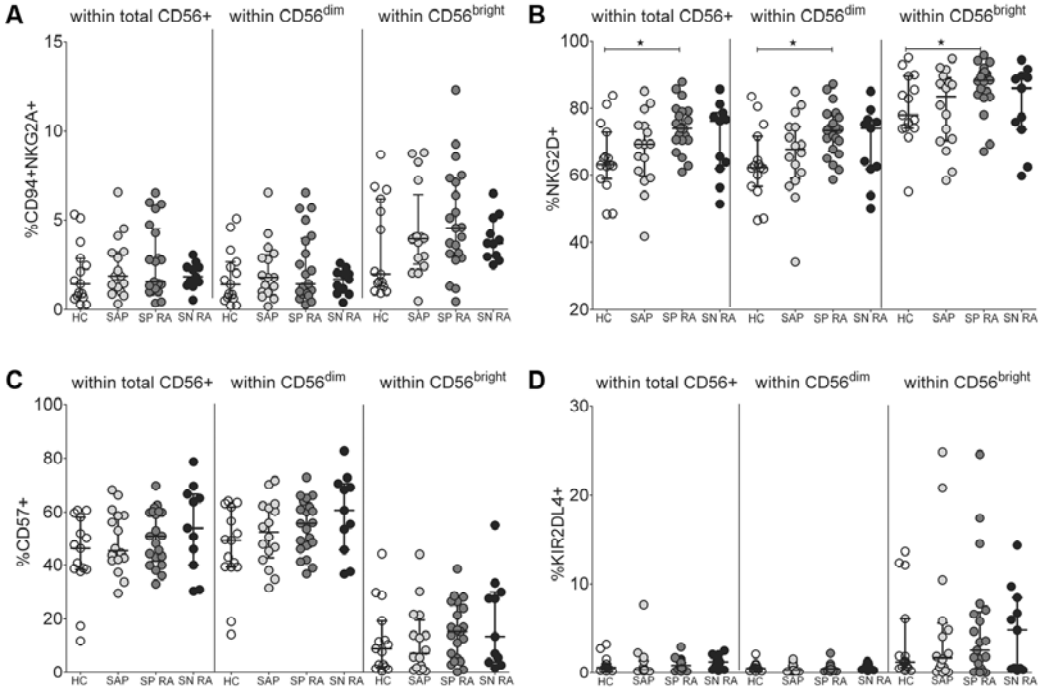
Also, our results demonstrate differences in the systemic immune profile between seropositive and seronegative RA adding to the notion that SP RA and SN RA may represent different disease entities.

Supplementary Figure 1. Numbers of circulating T-cells and B-cells in patients and controls.



Absolute numbers of (A) CD3+CD4+ T-cells, (B) CD3+CD8+ T-cells, (C) CD19+ B-cells and the relative frequencies within CD45+ lymphocytes of (D) CD3+CD4+ T-cells, (E) CD3+CD8+ T-cells and (F) CD19+ B-cells was assessed in the peripheral blood of HC (n=33), SAP (n=30), early RA SP (n=44) and SN RA (n=11). Statistical significance: * p <0.05.

Supplementary Figure 2. CD56^{dim} and CD56^{bright} NK-cell subsets from SP RA are characterized by an increased NKG2D expression.



Frequencies of (A) CD94+NKG2A+, (B) NKG2D+, (C) CD57+ and (D) KIR2DL4+ cells within total CD56+, CD56^{dim} and CD56^{bright} NK-cell subsets was assessed using PBMC from HC (n=15), SAP (n=16), early SP RA (n=20) and SN RA (n=11). Statistical significance: * p <0.05.

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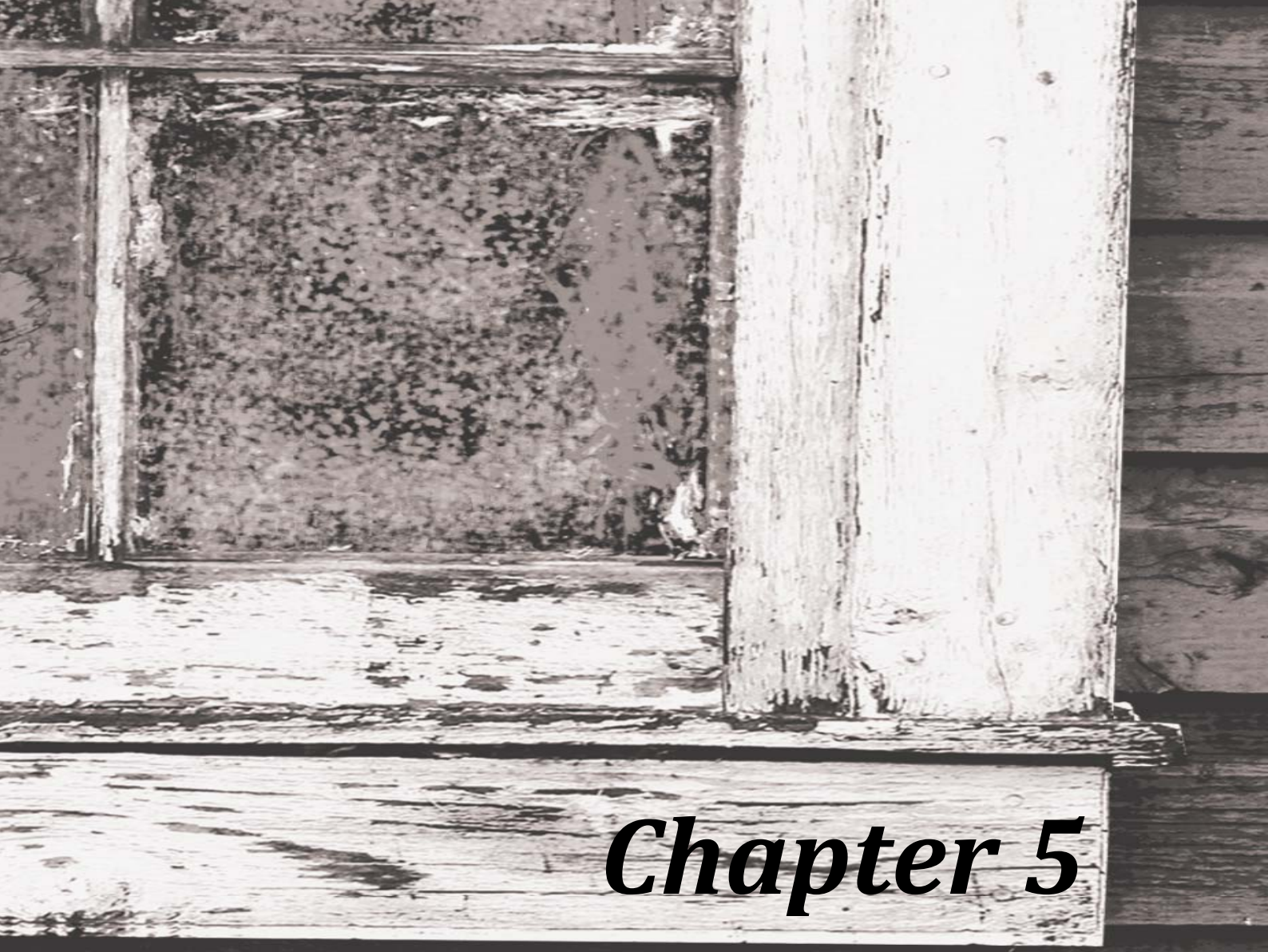

Chapter 5

Circulating CD4+CD161+ T lymphocytes are increased in seropositive arthralgia patients but decreased in patients with newly diagnosed rheumatoid arthritis

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Abstract

Improved understanding of the immune events discriminating between seropositive arthralgia and clinical synovitis is of key importance in rheumatology research. Ample evidence suggests a role for Th17 cells in rheumatoid arthritis. We hypothesized that CD4+CD161+ cells representing Th17 lineage cells may be modulated prior to or after development of clinical synovitis. Therefore, in a cross-sectional study, we investigated the occurrence of CD4+CD161+ T-cells in seropositive arthralgia patients who are at risk for developing rheumatoid arthritis and in newly diagnosed rheumatoid arthritis patients. In a prospective study, we evaluated the effect of methotrexate treatment on circulating CD4+CD161+ T-cells. Next, we assessed if these cells can be detected at the level of the RA joints.

Precursor Th17 lineage cells bearing CD161 were found to be increased in seropositive arthralgia patients. In contrast, circulating CD4+CD161+T-cells were decreased in newly diagnosed rheumatoid arthritis patients. The decrease in CD4+CD161+ T-cells correlated inversely with C-reactive protein and with the 66 swollen joint count. Methotrexate treatment led to normalization of CD4+CD161+ T-cells and reduced disease activity. CD4+CD161+ T cells were readily detected in synovial tissues from both early and late-stage rheumatoid arthritis. In addition, synovial fluid from late-stage disease was found to be enriched for CD4+CD161+ T-cells. Notably, synovial fluid accumulated CD4+CD161+T-cells showed skewing towards the Th1 phenotype as evidenced by increased interferon-γ expression.

The changes in peripheral numbers of CD4+CD161+ T-cells in seropositive arthralgia and early rheumatoid arthritis and the enrichment of these cells at the level of the joint predict a role for CD4+CD161+ T-cells in the early immune events leading to clinical synovitis. Our findings may add to the development of RA prediction models and provide opportunities for early intervention.

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by joint inflammation of synovial tissue eventually leading to cartilage and bone destruction. Early and combination treatment strategies were shown to be effective in controlling joint inflammation and prevention of bone erosions (1). Thus, early recognition of RA and identification of individuals at risk for arthritis development would open opportunities for early clinical intervention.

Autoantibodies such as rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA or anti-CCP) can be found in individuals already years before clinical synovitis becomes manifest. In a prospective study design, arthritis development in arthralgia patients was found to be associated with anti-CCP status (2,3). Of this seropositive arthralgia patient (SAP) group, 35% developed arthritis after a median follow-up period of 12 months (3). The majority of these patients (92%) fulfilled the 2010 American College of Rheumatology (ACR) criteria for classification of RA.

Understanding the immune events involved in the transition to clinical synovitis, and translation of these insights into the development of biomarkers is key (4). Ample evidence suggests a role for Th17 cells in the development of RA (5). In recent years, evidence was provided for the involvement of Th17 cells in the initiation phase of RA. First, the development of a cytokine environment favoring Th17 generation is an early event in RA pathogenesis (6). Second, in line with this, pre RA patients were found to show increased serum levels of IL-17 prior to the manifestation of clinical synovitis but these levels dropped significantly following the transition to RA (7). Although Th17 cells were detected in rheumatoid synovial tissue, Th17 cells appear to be outnumbered by interferon (IFN)-γ-producing Th1 cells at the level of the inflamed joint (8,9). The latter may be explained by reprogramming of Th17 cells to Th1 cells by synovial fluid derived IL-12, as shown in juvenile idiopathic arthritis (10,11).

Human CD161 is the homologue of mouse NK cell receptor-P1A and constitutes a type II transmembrane glycoprotein with characteristics of the C-type lectin superfamily (12). CD161 is now considered a marker of Th17 lineage cells (13-15). All human IL-17 producing cells are thought to originate from CD161+ precursors in umbilical cord blood and newborn thymus (13). CD161 expression is induced by RAR-related orphan receptor C (RORC), the Th17 lineage transcription factor (16). It seems that CD161 expression is maintained throughout the life cycle of the cell since it is detectable on memory circulating and tissue infiltrating Th17, Th17/Th1 and Th1 lymphocytes. CD161 expression thus presents a means to determine Th17 ancestry in peripheral and tissue infiltrating CD4+ T-cells (17,18).

We hypothesized that CD4+CD161+ T-cells representing Th17 lineage cells are involved in the initiation phases of RA and that peripheral numbers of these cells may be modulated prior to or after the development of clinical synovitis. Therefore, in a cross-sectional study, we investigated the occurrence of CD4+ CD161+ T-cells in SAP, newly diagnosed, treatment-naïve RA patients at baseline and at 3 and 6 months after start of methotrexate (MTX) treatment. Next, we assessed if CD4+CD161+ T-cells can be detected in early and late-stage RA synovial tissue. In late-stage RA, paired samples of peripheral blood

and synovial fluid were studied. Our study revealed altered numbers of peripheral CD4+CD161+ T-cells in SAP versus early RA patients and a relative enrichment of these cells in RA joints. The data suggest a role for CD4+CD161+ T-cells in the early immune events leading to clinical synovitis.

Materials and Methods

Ethics statement

Rheumatoid arthritis (RA) and seropositive arthralgia patients (SAP) gave their written informed consent. Twentyfour healthy control (HC) volunteers were recruited in 2010 and 2011 for establishing reference values on leukocyte subsets and T cell cytokine production. Documented oral (n = 20) or written informed consent (n = 4) was obtained. Documented oral consent and anonymisation of HC blood samples by a registered medical immunologist was approved by the Institutional Review Board. At time of blood sampling, healthy volunteer donors were requested to fill out a health questionnaire. All procedures were in accordance with institutional guidelines and approved by the local medical ethics committee (UMCG Groningen, the Netherlands). METC numbers: 2007.148, 2009.118, 2011.306 and 2012.375.

Study populations

Starting February 2012, twenty-six patients with a positive anti-CCP and/or IgM-rheumatoid factor (RF) status and (a history of) arthralgia, but not arthritis, defined as SAP, were recruited at the rheumatology outpatient clinic at the UMCG. Absence of arthritis was confirmed by physical examination of 44 joints by a trained medical doctor and a senior rheumatologist.

Thirty-five newly diagnosed, treatment-naive RA patients were recruited starting in 2010. All patients fulfilled the ACR 1987 or 2010 criteria (19,20) with a mean duration of preceding symptoms of 10,2 ± 16,5 months. Twenty-six and 12 RA patients were followed prospectively for a period of 3 (14 ± 3 weeks) and 6 months (29 ±7 weeks) respectively after start of MTX treatment.

Paired samples of peripheral blood (PB) and synovial fluid (SF) were obtained from 11 RA patients with late-stage RA. All of these patients fulfilled the ACR 1987 criteria and were treated with different disease modifying anti-rheumatic drugs (DMARDs, e.g. MTX, Etanercept, Adalimumab and Prednisolone).

Twenty-four healthy subjects, matched for age, sex and ethnicity, were included as a control group. Exclusion criteria were inflammation, malignancy (past or present), or use of immune suppressive drugs.

Demographic and clinical characteristics of patients and controls are summarized in Table 1. In addition, synovial tissues (ST) were obtained from 15 early, treatment naïve RA patients undergoing knee or ankle arthroscopy. Four out of 15 biopsy tissues contained clear cellular infiltrates and were processed for immunohistochemistry (IHC, n = 4. 3 out of 4 were female, median age of 60.5 (range 53,2 - 67,8), 3 out of 4 were anti-CCP positive and all 4 were RF positive). In addition, we obtained ST biopsies from 4 RA patients with late-stage, active RA (4/4 were female with a mean age of 54,0± 4,4; C-reactive protein

(CRP) median value (range) of 24,0 mg/L (7,0-63,0); erythrocyte sedimentation rate (ESR) median value (range) of 48,5 mm/h (19,0-51,0); 1/4 were anti-CCP-positive; 3/4 were RF-positive).

Table 1. Demographic and clinical characteristics of subjects included in the study.

	HC	SAP	Longitudinal study			Late-Stage RA
			Early RA Baseline	Early RA 3 months MTX	Early RA 6 months MTX	
N	24	26	35	26	12	11
Age (yrs); mean ± SD	58.8 ±10.4	55.2 ±10.5	59.9 ±10.9	59.7 ±11.5	56.9 ±9.7	62.1 ±12.5
Gender; % female	79.2	65.4	80.0	77.0	42.0	63.6
SE-containing HLA-DRB1 alleles; % positive	66.7	66.7	70.0	81.2	100	nd
Disease duration (yrs); median (range)	na	na	na	na	na	5.8 (2.4-43.9)
Erosive (%) X ray	na	na	20.0	nd	33.3	63.6
CRP (mg/L); median (range)	na	5.0 (5-29)	14.0 (5-118)*	5.0 (5-45)†	5.0 (5-16)†	21.5 (5-49)* #
ESR (mm/h); median (range)	na	14.0 (2-69)	26.0 (2-96)*	18.0 (5-76)	12.0 (4-55)	23.5 (3-80)
RF; % positive	na	73.1	74.3	76.9	83.3	63.6
Anti-CCP; % positive	na	96.0	68.6	73.1	75.0	90.9
DAS28; mean ± SD	na	na	5.1 ±1.3	3.7 ±1.3†	2.8 ±1.0†	2.4 ±1.0†

SE = shared epitope (SE-containing alleles are DRB1*0401, *0404, *0405, *0408, *0101, *0102 and *1001); CRP= C-reactive protein; ESR= erythrocyte sedimentation rate; RF= rheumatoid factor (positive score defined as ≥ 15 IU/mL); Anti-CCP= anti– cyclic citrullinated peptides antibodies (positive score defined as > 10 IU/mL); DAS28= disease activity score 28; nd = not determined; na = not applicable. * $p < 0.05$ compared to seropositive arthralgia patients; † $p < 0.05$ compared to RA patients at baseline; # $p < 0.05$ compared to RA patients after 3 mo MTX treatment.
Seropositive arthralgia patients (SAP) inclusion criteria were anti-CCP and /or RF seropositivity and arthralgia affecting at least one joint.

Anti-CCP and RF determination

Anti-CCP serum levels were determined by anti-IgG CCP fluorescent enzyme immuno assay on Phadia 250 (Thermo Fisher Scientific, Uppsala, Sweden). RF total Ig serum levels were determined by turbidimetry using modular analyzer (Roche, Mannheim, Germany). Seropositive status was defined as anti-CCP serum levels of ≥ 10 IU/mL and/or RF serum levels of ≥ 15 IU/mL.

Flowcytometric analysis of T-cell-surface markers and cytokine expression

The absolute numbers and the phenotype of lymphocytes in PB and SF were determined using multicolor flow cytometry. Briefly, 100 µl of EDTA anti-coagulated PB or SF was collected and stained, according to the manufacturers instructions, with combinations of the following antibodies: anti-CD4 PerCP, anti-CD8 PerCP, anti-CD45RO FITC, anti-CCR7 PE, anti-CD161 APC (BD Biosciences, San Jose, CA, USA) or anti-CD3 eFluor 605NC, anti-CD4 *eFluor450 (*eBioscience, San Diego, CA, USA), CD8

PerCP, anti-CD45RO FITC, anti-CCR7 PE-Cy7 (BD Biosciences), anti-CD161 PE (Miltenyi Biotec, Leiden, The Netherlands).

Absolute numbers of leucocytes, CD3 and CD4 T-cells were determined using the BD MultiTest TruCount method with six-color MultiTest reagents detecting CD45, CD3, CD4, CD8, CD19, CD16+CD56 (BD) using a lyse-no-wash preparation method as described by the manufacturer. Flowcytometry was performed on FACSCanto II (BD) and analysis was performed using FACSCanto Clinical Software (BD). CD4+ T-cells were further characterized by analyzing expression of CCR7 and CD45RO (21). Percentages of T lymphocytes with a naïve (T_{Naive}), central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated (T_{EMRA}) phenotype within the total CD4 counts were used to calculate the absolute numbers of these subsets.

To assess the potential of CD4+ CD161+ T-cells to produce cytokines, 100 µl of heparinized PB or 100 µl of SF was stimulated with phorbol myristate acetate (PMA) (50 ng/mL) and ionomycin (1,6 µg/mL) in the presence of brefeldin A (BFA) (10 µg/mL) (Sigma–Aldrich, Zwijndrecht, The Netherlands) for 4 h at 37°C. After stimulation, erythrocytes were lysed with ice-cold ammonium chloride buffer (pH 7,4 for 10 min on ice). Cells were fixed and permeabilized using Fix & Perm Cell Permeabilization Reagents (Life Technologies, Bleiswijk, The Netherlands) and stained with anti- IFNγ PerCP-Cy5.5, anti-tumor necrosis factor (TNF)-α PerCP-Cy5.5 (BioLegend, San Diego, CA, USA), anti-CD3 eFluor605NC, anti-CD4 eFluor450, anti-IL-17 FITC, Perforin FITC *(*eBioscience*),* anti-CD8 APC-H7 (BD) and anti-CD161 APC (Miltenyi Biotec*).* Mouse isotype controls were used in parallel at the same concentrations. Cells were analyzed using FACS Calibur and LSR II flowcytometers (BD Biosciences), and data analysis was performed with FlowJo™ software (Tree Star, Ashland, OR, USA).

Synovial tissue and IHC

ST biopsies, obtained from 15 newly diagnosed, treatment-naïve RA patients undergoing knee or ankle arthroscopy were snap frozen and stored at -80 °C. Biopsy sections were first analysed for the presence of cellular infiltrates. Clear infiltrates were detected in 4 out of 15 patient biopsies. Positive biopsies were examined for the presence of CD161-expressing T cells. Cryostat sections (5 µm) were cut and stored at -20 °C. Air-dried sections (30 min) were fixed with 3% paraformaldehyde and 0.3% glutaraldehyde for 10 min. Sections were incubated with 0.1% hydrogen peroxide (Merck, Amsterdam, The Netherlands) for 10 min or Avidin/Biotin Blocking kit (Vector, Burlingame, CA, USA) according to the manufacturers instructions. Following washing, sections were incubated with the primary antibodies: anti-CD161 (polyclonal rabbit anti-human CD161 from Sigma-Aldrich) diluted 1:20, anti-CD3 (monoclonal mouse anti-human CD3 from Dako, Glostrup, Denmark,) diluted 1:50, anti-CD4 (monoclonal mouse anti-CD4 from Lifespan BioSciences, Seattle, WA, USA) diluted 1:10 in PBS for 1 hr at RT. Following washing, secondary donkey anti-rabbit-alkaline phosphatase (AP) antibody diluted 1:200, goat anti-mouse IgG1-biotin antibody diluted 1:100 and goat anti-mouse IgG2a-HRP antibody diluted 1:200 (SouthernBiotech,

Birmingham, AL, USA) were used respectively. After incubation for 1 hr, CD3 stained tissue sections were incubated with streptavidin-AP (SouthernBiotech) for 30 min at RT. AP substrate (Sigma-Aldrich) or HRP (Dako) were used according to the manufacturers instructions. Tissue sections were counterstained with hematoxylin (Merck).

Synovial tissue and cell isolation

ST biopsies, obtained from late-stage RA patients undergoing hip or knee joint replacement surgery, were rinsed with Dulbecco's modified Eagles Medium (DMEM, Life Technologies), supplemented with 60 μg/mL gentamycin (Life Technologies) and cut into small fragments using a scalpel. The minced tissue was then incubated with 80 units/mL of hyaluronidase from bovine testes (Sigma-Aldrich) at 37°C. After 15 min. collagenase type IV from *Clostridium histolyticum* (Life Technologies) was added (final concentration of 1 mg/mL). Tissue was further digested, while rotating (225 rpm) for 2 hr at 37°C. After filtration and washing cells were stained with the following antibodies: anti-CD3 eFluor605NC, anti-CD4 eFluor450 (eBioscience), anti-CD19 PerCP, anti-CD161 PE (BD).

Statistical analysis

Power analysis for the sample size calculation was performed based on the results obtained from a pilot experiment involving 20 RA patients and 20 HC. A 2-sided power analysis was done with a confidence interval and power of 95% and 90%, respectively. Results are expressed as mean ± standard deviation (SD) or median (range) for normally distributed and non-normally distributed data, respectively. Normally distributed data was analyzed using ANOVA and unpaired t test. Non-normally distributed data was analyzed using Kruskal-Wallis test and Mann-Whitney 2-tailed test. Correlations between cell numbers and clinical data were evaluated by nonparametric Spearman's correlation analysis. Paired samples analysis was performed with Wilcoxon signed rank test. Generalized estimating equations (GEE) analysis was used to analyze parameters over time within patients. Simple contrasts were used to compare follow-up visits to baseline. Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics 20 (SPSS, Chicago, IL, USA). $P<0.05$ was considered statistically significant.

Results

Circulating effector memory cells are increased in seropositive arthralgia patients when compared to newly diagnosed RA

To identify immune markers associated with arthralgia and/or clinical synovitis, we first assessed if the peripheral leukocyte pool was altered in SAP and in newly diagnosed, DMARD-free RA patients (when compared to healthy controls). Absolute numbers of leukocytes, total T-cells and CD4+ T-cells were comparable between groups (Table 2). Next, CD4+ T-lymphocytes with a naïve (T_{Naive}), central memory

(T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory (T_{EMRA}) phenotype, defined by expression of CD45RO and CCR7, were assessed. Absolute numbers of T_{Naive}, T_{CM}, T_{EM} and T_{EMRA} in SAP were similar to healthy controls. No statistically significant differences were noted. Interestingly, absolute numbers of T_{EM} and T_{EMRA} were significantly elevated in SAP when compared to RA. In addition, absolute CD4+ T_{Naive} counts tended to be increased in SAP and were significantly increased in newly diagnosed RA patients when compared to HC. The latter phenomenon is in line with previous reports on expansion of naïve phenotype cells in RA (22).

Table 2. Absolute numbers of circulating leukocyte subsets in HC, SAP and RA patients.

	HC	**SAP**	**RA Baseline**
N	20	26	30
CD45+ cell absolute count x10^6/mL, median (range)	1.89 (1.25-2.63)	1.91 (0.89-12.3)	1.86 (1.31-3.01)
CD3+ cell absolute count x10^6/mL, median (range)	1.34 (0.93-1.80)	1.42 (0.51-3.63)	1.41 (0.83-2.50)
CD3+CD4+ cell absolute count x10^6/mL, median (range)	0.96 (0.49-1.29)	1.00 (0.39-2.44)	0.90 (0.50-1.68)
CD4+ Naïve (CD45RO-CCR7+) cell absolute count x10^6/mL, median (range)	0.25 (0.09-0.58)	0.36 (0.07-1.45)	0.40 * (0.15-0.95)
CD4+ Central Memory (CD45RO+CCR7+) cell absolute count x10^6/mL, median (range)	0.28 (0.03-0.39)	0.28 (0.12-0.84)	0.28 (0.08-0.72)
CD4+ Effector Memory (CD45RO+CCR7-) cell absolute count x10^6/mL, median (range)	0.26 (0.13-0.58)	0.31 † (0.11-0.53)	0.25 (0.11-0.49)
CD4+ Terminally Differentiated EM (CD45RO-CCR7-) cell absolute count x10^6/mL, median (range)	0.020 (0.004-0.56)	0.034 † (0.005-0.23)	0.018 (0.004-0.14)

* $p < 0.05$ RA patients at baseline vs HC; † $p < 0.05$ Seropositive arthralgia patients (SAP) vs RA patients at baseline (Mann-Whitney test). The absolute numbers of CD45+, CD3+, CD3+CD4+ lymphocytes were determined using the BD MultiTest TruCount method. Relative values (%) of T lymphocytes with a naïve (TNaive), central memory (TCM), effector memory (TEM) and terminally differentiated effector memory (TEMRA) phenotype within the total CD4 counts were used to calculate the absolute numbers of these subsets.

Circulating CD4+CD161+ T lymphocytes are increased in seropositive arthralgia patients but decreased in patients with newly diagnosed RA

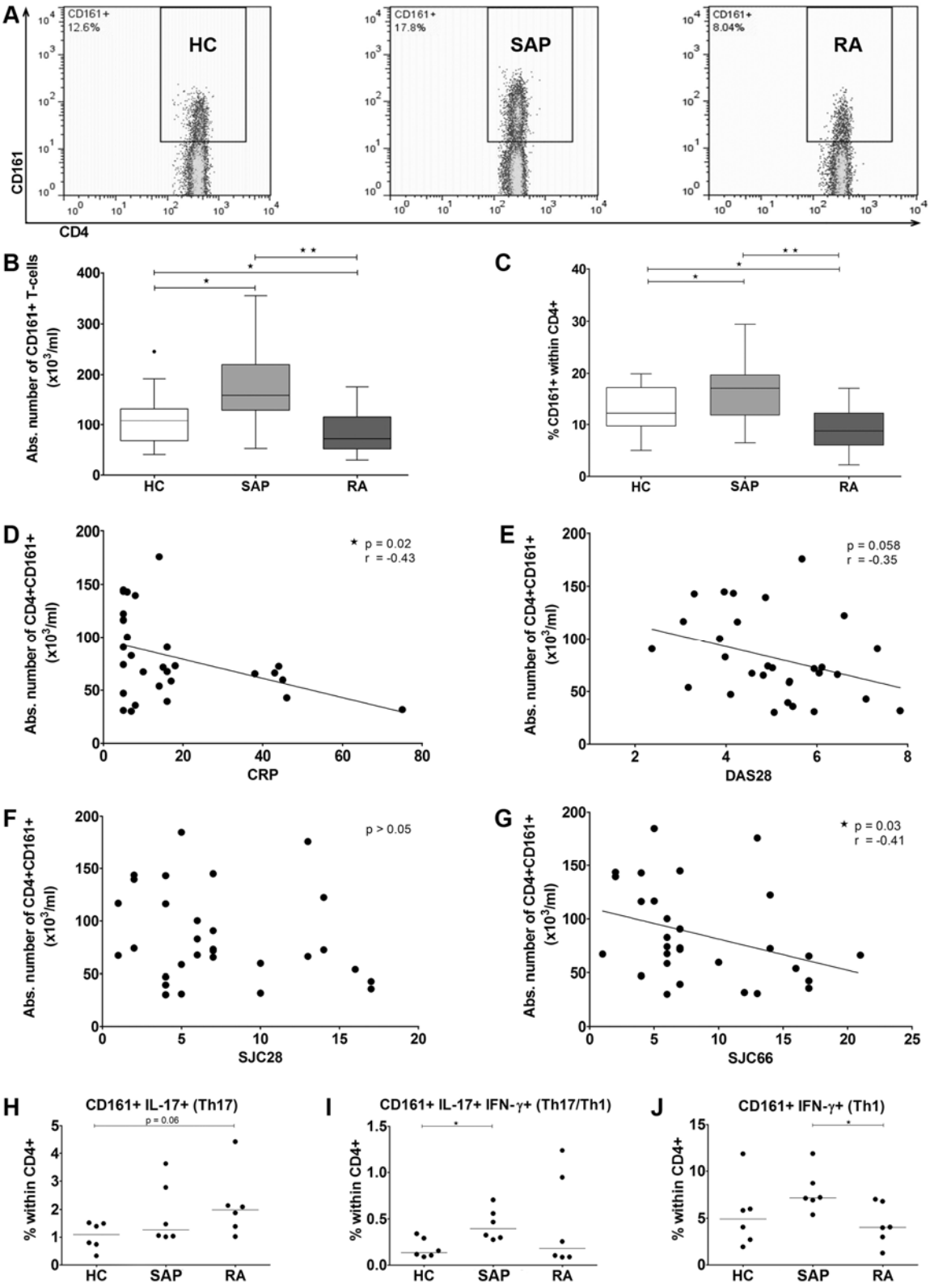
We next investigated circulating T-cells expressing the Th17 lineage marker CD161 in SAP and RA patients. Whereas absolute numbers of CD4+CD161+ T-cells were found increased in SAP, a significant decrease of these cells was noted in newly diagnosed RA patients (Fig 1A, B). Similar results were obtained when proportions of CD161+ T-cells within the CD4 subset were calculated (Fig 1C). Next, we assessed whether the decrease of the CD4+CD161+ population was associated with clinical measures of disease activity. The absolute numbers of CD4+CD161+ T-cells as seen in newly diagnosed RA correlated inversely with CRP ($r = -0,43$ and $P = 0.02$, Fig 1D). Moreover, CD4+CD161+ T-cells tended also to correlate inversely with the disease activity score 28 (DAS28, $r = -0,35$ and $P = 0.058$, Fig 1E). Absolute numbers of CD4+CD161+ cells did not correlate with the 28 swollen joint count (SJC) ($r = -0,26$ and $P = 0.18$, Fig 1F) in which ankles and feet are not included. Interestingly, absolute numbers of CD4+CD161+ cells were found to correlate inversely with the total 66 SJC ($r = -0,41$ and $P = 0.03$, Fig 1G).

Previously, CD4+CD161+ T-cells were found to contain Th17 (defined by expression of IL-17 but not IFN-γ) and two progeny subsets: Th17/Th1 (defined by expression of both IL-17 and IFN-γ) and Th1 (the so-called non classical Th1 defined by expression of IFN-γ but not IL-17) (17,18). We assessed the relative frequencies of these cells by analyzing the CD4+CD161+ T-cell cytokine producing potential in the different groups (Fig 1H-J). Frequencies of circulating Th17 cells tended to be increased in newly diagnosed RA when compared to HC (Fig 1H). Th17/Th1 double positive cells were found increased in SAP (Fig 1I). Non classical Th1 were found to be decreased in newly diagnosed RA when compared to the SAP group (Fig 1J).

Circulating CD4+CD161+ T-cells normalize following treatment

Newly diagnosed RA patients were assessed at baseline (before start of MTX treatment) and at 3 and 6 months after start of treatment for absolute numbers of circulating CD4+CD161+ T-cells and for clinical parameters of disease activity. MTX treatment significantly reduced CRP and Disease Activity Score (DAS)28, but not ESR, at 3 and 6 months when compared to baseline (Table 1). Importantly, the reduction in CRP and DAS28 was associated with an increase in the absolute number of circulating CD4+CD161+ T-cells (Fig. 2A, B). Notably, the numbers of PB CD4+CD161+ T-cells increased to the level observed in healthy subjects at 3 and 6 months (Fig. 2 C). The data merit further study into the utility of circulating CD4+CD161+ T-cells as a potential biomarker of synovitis in RA.

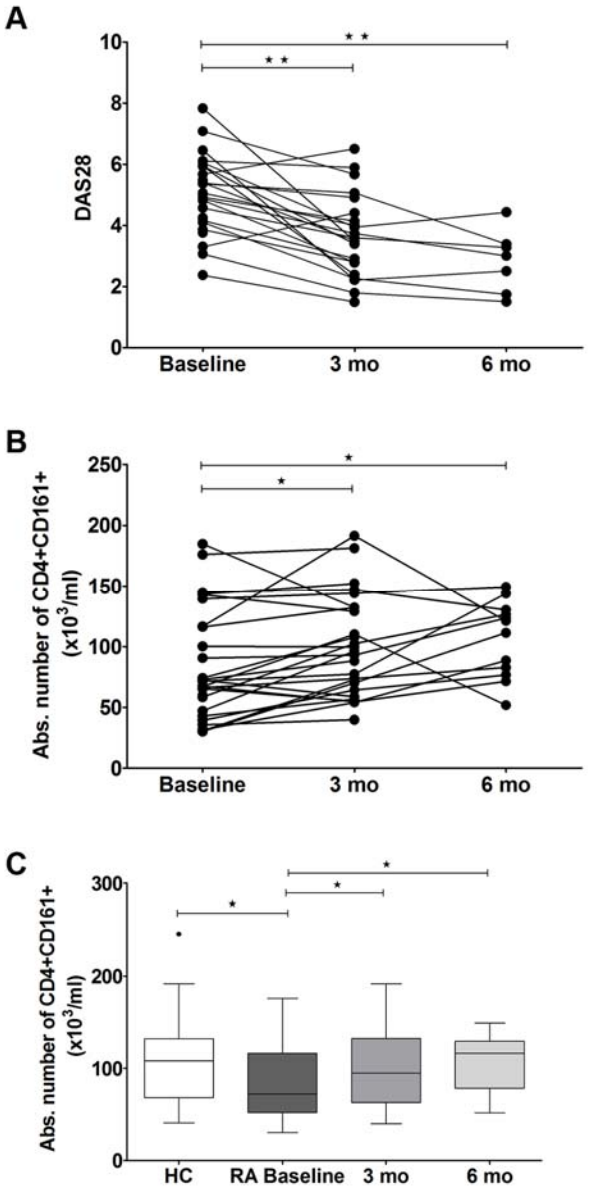
Figure 1. Altered dynamics of circulating CD4+CD161+ T-cells in seropositive arthralgia patients and in newly diagnosed RA patients.



(A) Representative dot plots showing proportions of CD4+CD161+ T-cells in the study groups. The absolute number (B) and the frequency (C) of CD161+ cells within CD4+ T-cells from healthy donors (n=20), SAP (n=26) and early RA patients (n=35; Mann-Whitney test). Horizontal line in the box represents the median value. Boxes represent interquartile range and whiskers represent the actual range. Symbols outwith the boxes represent outliers. The correlation between the absolute number of CD161+ cells within CD4+ T-cells and (D) CRP (mg/l), (E) DAS28, (F) SJC28 and (G) SJC66 in RA patients (n=30; Spearman coefficient analysis). Frequency of CD4+CD161+ -cells expressing (H) IL-17, (I) IL-17 and IFN-γ or (J) IFN-γ alone within total CD4+ from HC (n=6), SAP (n=6) and early RA patients (n=6;

Mann-Whitney test). Statistical significance is indicated as * $p \leq 0.05$, ** $p \leq 0.001$, CRP= C-reactive protein, DAS= disease activity score, SJC= swollen joint count.

Figure 2. Circulating CD4+CD161+ T-cells normalize following MTX treatment



(A) DAS28 and (B) the absolute number of CD4+CD161+ T-cells from RA patients at baseline (newly diagnosed; n=30), after 3 months (n=22) and 6 months (n=7) of MTX treatment (GEE analysis); (C) Comparison of the absolute number of CD4+CD161+ T-cells between HC (n=20) and RA patients at baseline (n=30; Mann-Whitney test) or RA patients at baseline (n=30) and RA patients after 3 months (n=26) or 6 months (n=12) of MTX treatment (Wilcoxon matched pairs test). Horizontal line in the box represents the median value. Boxes represent interquartile ranges and whiskers represent the actual range. Statistical significance is indicated as * $p \leq 0.05$, ** $p \leq 0.001$.

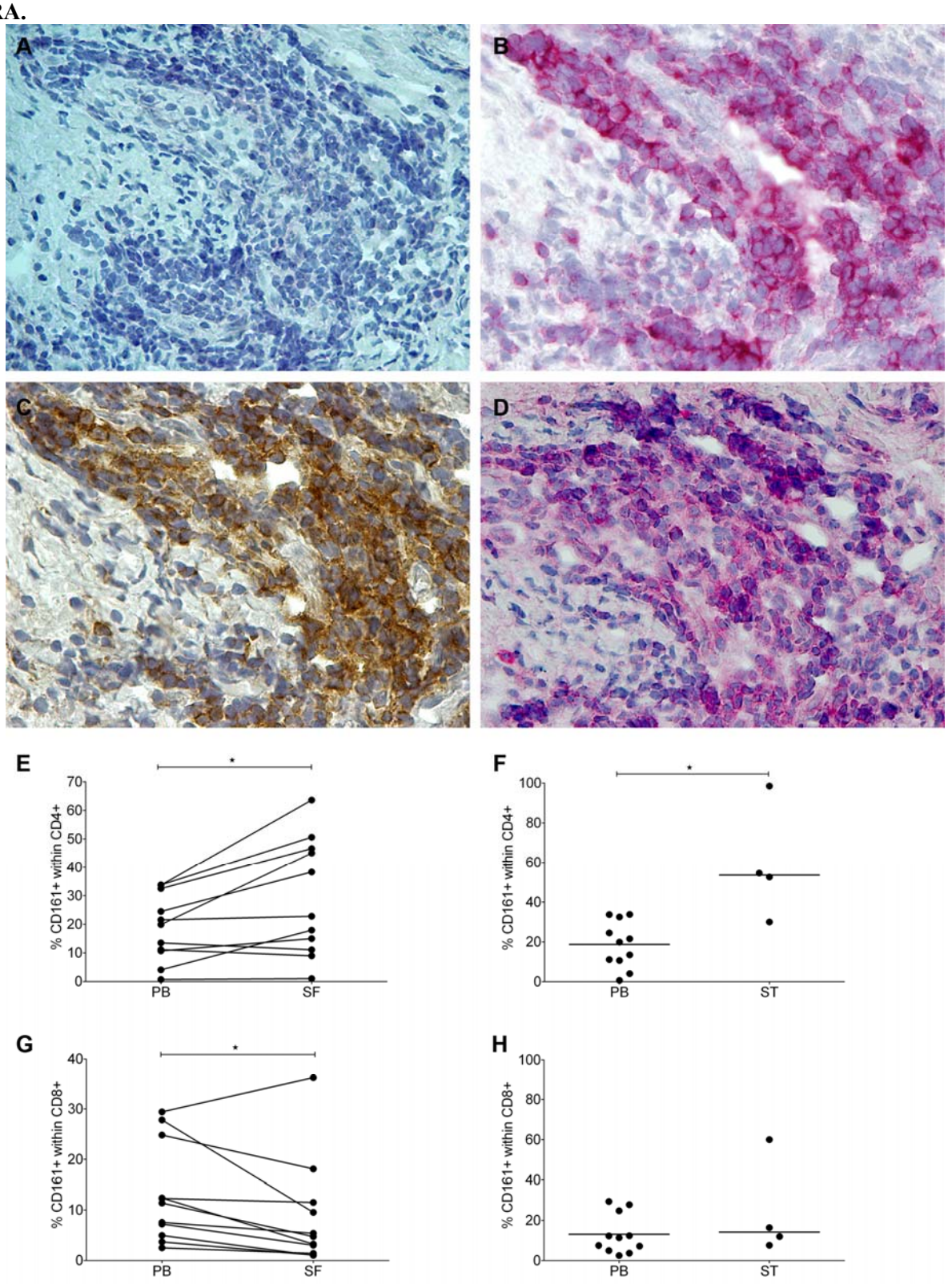
CD4+CD161+ T-cells are found at inflamed sites in RA joints

CD161 may function as an adhesion molecule and thereby facilitate migration (14,23). To assess if the observed decrease of circulating CD4+CD161+ T-cells in newly diagnosed RA patients may be explained by their homing to the site of inflammation, we analyzed CD161 expression in ST samples obtained via arthroscopy in this group. CD4+ CD161+ T-cells were readily detected in ST sections using IHC. Representative staining of consecutive synovial biopsy sections showed clear staining for CD161 in the area's infiltrated by CD3- and CD4- expressing cells (Fig 3A-D).

Next, we investigated CD4+CD161+ T-cells in patients with late-stage disease. To that end, relative frequencies of these cells were assessed in paired samples of PB and SF. Also, we assessed the presence of CD4+CD161+ T-cells in digested ST biopsies by flowcytometry. The frequency of CD4+CD161+ T-

cells in the SF was significantly increased when compared to PB (mean value 29,2% within the total CD4+ in SF vs. 18,7% within total CD4+ in PB, Fig. 3E). Similarly, flowcytometric analysis of digested ST from late-stage RA showed an increased frequency of CD4+CD161+ T-cells (median value 58,9% of total CD4+ in ST, Fig 3F). In contrast, we did not observe an accumulation of CD8+CD161+ T-cells in SF or ST (Fig. 3G, H).

Figure 3. CD4+CD161+ T-cells are readily detected at the level of the joint in newly diagnosed and in late-stage RA.

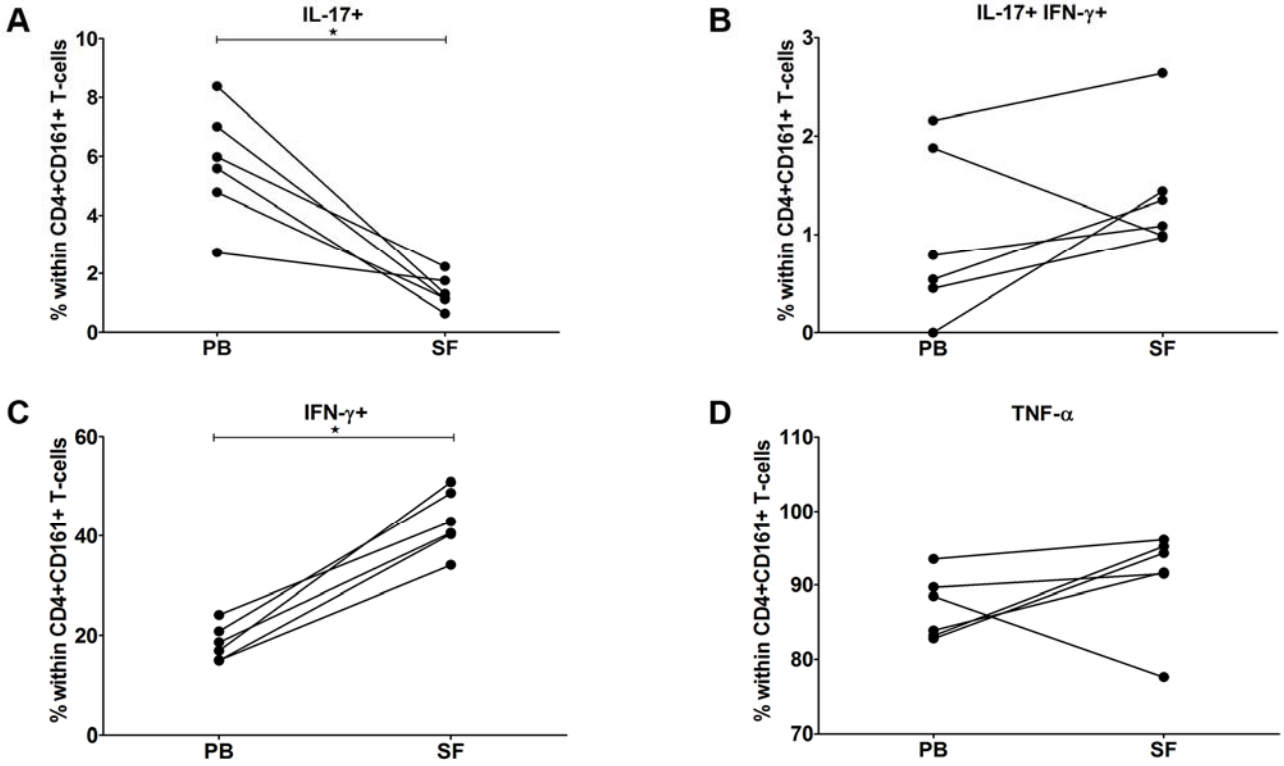


Detection of T cells expressing CD161 in ST obtained from a newly diagnosed RA patient using IHC on consecutive cryostat sections (Krenn score = 4 (37)). A representative example is shown (magnification 40x). Blanc (A), CD3 (B), CD4 (C), CD161 (D). Analysis of the number of CD4+CD161+ T-cells from paired PB and SF and non-paired PB and ST from late-stage RA. Frequency of CD161+ cells within (E, F) CD4+ and (G, H) CD8+ T-cells were compared between paired samples of PB and SF (n=6; Wilcoxon matched pairs test) or PB and enzyme-digested ST (n=4; Mann-Whitney test).

Synovial fluid-derived CD4+CD161+ T-cells show enhanced IFN-γ-producing capacity

Since the CD4+CD161+ T-cells were detected at the level of the joints, we further investigated the Th17, Th17/Th1 and Th1 phenotypes and analyzed their cytokine producing potential in paired SF and PB samples from RA patients with late-stage disease. Interestingly, the percentage of IL-17-producing cells was significantly higher within the PB-derived CD4+CD161+ subset than the SF-derived subset (median value 5,1% vs. 1,6% within CD4+CD161+ in PB and SF, respectively, Fig. 4A), while the capacity for production of both IFNγ and IL-17 was similar for PB- and SF- derived CD4+CD161+ T-cells (Fig 4B). Of note, a significant increase in the frequency of single IFNγ+ cells (non classical Th1) was seen in SF when compared to PB (median value 39,0% vs. 18,8%; Fig.4C). Expression of TNF-α was variable between CD4+CD161+ T-cells from PB and SF (Fig 4D). Thus, CD4+CD161+ T-cells in the joints of late-stage RA display a skewing towards a pro-inflammatory IFN-γ signature.

Figure 4. Synovial fluid CD4+CD161+ T-cells demonstrate a Th1 phenotype.



Paired samples of PB and SF from late-stage RA patients (n=6) were stimulated using PMA/ionomycin in the presence of BFA. The frequency of CD4+CD161+ T lymphocytes producing (A) IL-17, (B) both IL-17 and IFN-γ (C) IFN-γ, or (D) TNF-α was assessed (Wilcoxon matched pairs test). Statistical significance is indicated as * $p \leq 0.05$.

Discussion

A better understanding of the immune events in the switch to clinical synovitis would open opportunities for prevention of RA development (4). Thus, the definition of biomarkers discriminating between eropositive arthralgia and clinical synovitis is eagerly awaited. We report on profound changes in circulating precursor Th17 cells in SAP who are at risk of developing RA and in newly diagnosed patients with RA (before start of DMARD treatment). Whereas absolute numbers of CD4+CD161+ T-cells were found increased in the seropositive arthralgia group, a profound decrease of these cells was found to mark the early RA state. The decrease in the absolute number of CD4+CD161+ T-cells in early RA correlated inversely with CRP and with the SJC66. MTX treatment led to normalization of CD4+CD161+ T-cells and reduced disease activity.

Our findings add to earlier reports implicating Th17 cells in the initiation phase of RA. Indeed, a cytokine environment favoring Th17 generation is an early event in RA pathogenesis (6). Importantly, pre RA patients were found to show increased serum levels of IL-17 prior to the manifestation of clinical synovitis but these levels dropped significantly following the transition to RA (7). The reported drop in systemic IL-17 levels is mirrored by our observation on the dynamics of Th17 precursor cells expressing CD161 in SAP (increase) and in clinical synovitis (decrease). Moreover, the inverse correlation with the SJC66 would suggest their homing to the joint. Indeed, CD4+CD161+ T cells were readily detected in ST from both newly diagnosed and late-stage RA patients. In addition, synovial fluid from late-stage RA was found to be enriched for CD4+CD161+ T-cells which is in line with the effector memory phenotype of these cells ((21) and own observations). Migration of CD4+CD161+ T-cells to the joints is mechanistically explained by CCL20 induced migration. CCL20 expression in SF and ST has been reported previously to attract CCR6+ cells (24-26). Notably, CCR6 expression is a feature of CD4+CD161+ T-cells. Both CD161 and CCR6 expression were found to be Th17 lineage transcription factor (RORC) dependent (16). Transendothelial migration may be facilitated by CD161 mediated adhesion (23).

Interestingly, we found that the decrease in Th17 precursor cells was correlated with the SJC66 but not with the SJC28. The SJC66 includes ankles and feet, and thus provides a more comprehensive appreciation of joint involvement. This would be in line with earlier reports suggesting that the joints of the feet are important in very early RA (27).

In a prospective, longitudinal study, we demonstrated that peripheral CD4+CD161+ T-cell numbers normalize following regular MTX treatment. This may be explained by inhibition of migration due to MTX-mediated reduction of pro-inflammatory cytokines, chemokines and adhesion molecule expression in the joints (28,29). It is currently not know if MTX affects CCL20 production, the primary chemokine for Th17 lineage cells. Our data thus reveal profound effects of MTX treatment on peripheral numbers of CD4+CD161+ cells and call for caution when interpreting data on cellular immune markers in patients receiving immune suppressive treatment.

In this study, we examined the dynamics of Th17 precursor T-cells in 3 unique cohorts of patients: in SAP who are at risk of developing RA, in newly diagnosed RA patients before and after start of MTX treatment and in late-stage RA. Previously, Miao et al reported on increased relative frequencies of IL-17 producing CD4+CD161+ T-cells that correlate with disease activity in RA (30). This patient cohort had a mean disease duration of 3-4 years and most of these patients were treated with DMARDs. Thus, this study cohort can best be compared to our late-stage RA group on treatment. Although these authors did not report on absolute numbers of CD4+CD161+ cells, the reported percentages of IL-17 producing CD4+ CD161+ T-cells compare well with our data in late-stage RA (mean of 5% with ranges between 2-10%).

In late-stage RA, synovial fluid CD4+ CD161+T-cells showed skewing towards the Th1 phenotype when compared to peripheral blood CD4+CD161+ T-cells. This is in line with previous data reporting on Th17 plasticity towards Th17/Th1 and Th1 cells (10,11). The instability of the Th17 phenotype at the level of the joint may be explained by synovial fluid derived factors including IL-12 (11). Alternatively, the plasticity of Th17 lineage cells at the level of the joint is explained by other mechanisms involving ligation of CD161 with naturally occurring ligands. The only confirmed endogenous ligand for CD161 is lectin-like transcript 1 (LLT1) (31-33). LLT1 is expressed by activated antigen presenting cells and lymphocytes (34). Interestingly, CD161 cross linking in vitro was shown to facilitate IFNγ production by T-cells (31,34). Others reported on increased T-cell IL-17 production (35). There is currently no data available on expression of LLT1 in RA. More studies are needed to assess if CD161-LLT1 ligation relays co-stimulatory signals and if this contributes to Th17 function and or Th1 skewing at the level of the joint in RA.

The search for biomarkers characterizing the transition to clinical synovitis is eagerly awaited and would present opportunities for prevention of RA. Also, candidate biomarkers would add to prediction models that are currently being developed (3,36). Circulating Th17 lineage cells were increased in patients at risk for developing RA but decreased in newly diagnosed RA. MTX treatment led to normalization of circulating CD4+CD161+ T-cells. The decrease of CD4+CD161+ T-cells in early RA was associated with the SJC66. An improved mechanistic understanding of CD4+CD161+ T-cells in the switch to RA synovitis may ultimately provide novel treatment options.

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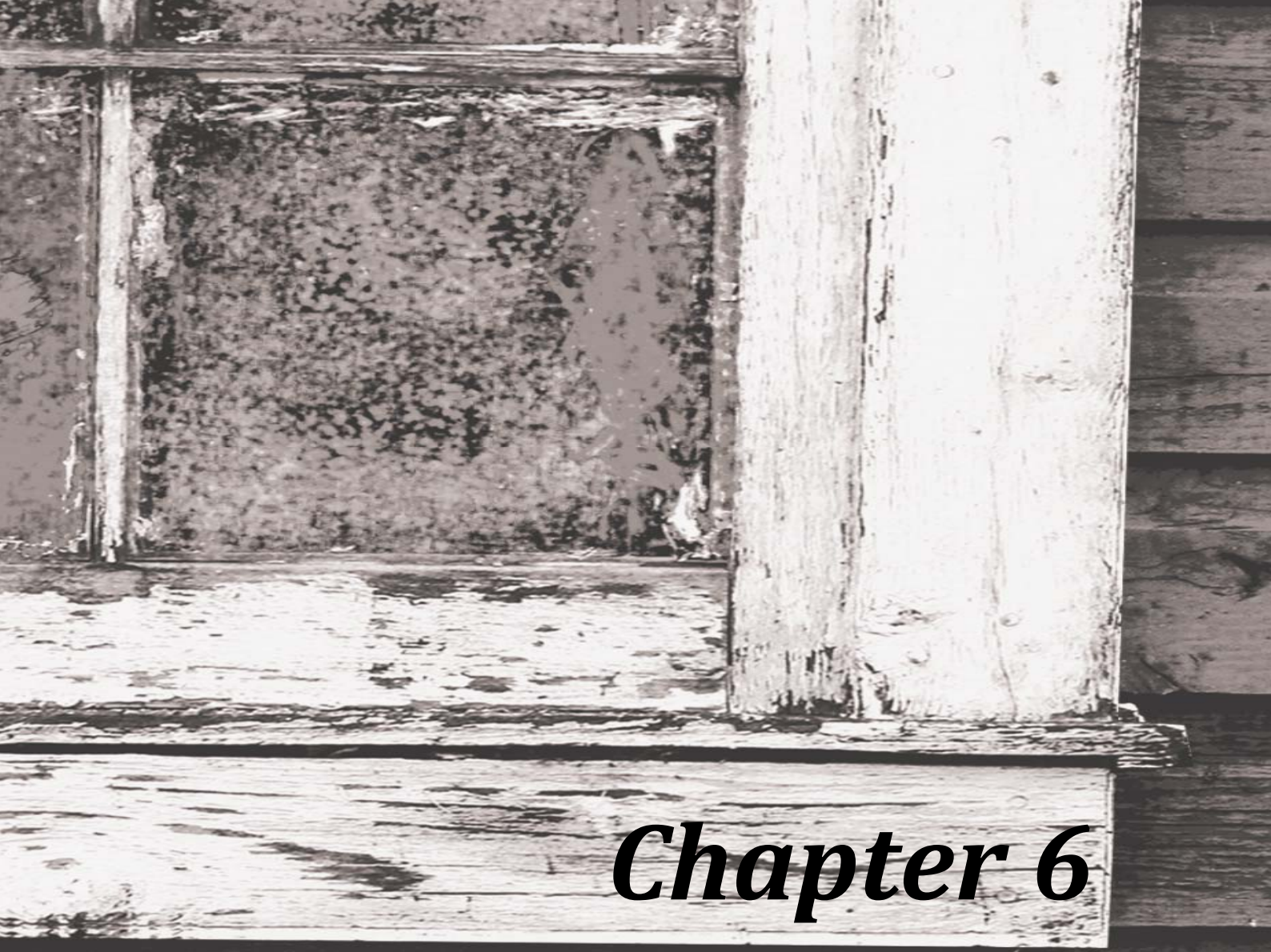

Chapter 6

Expression of Lectin-Like Transcript 1, the ligand for CD161, in rheumatoid arthritis

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Abstract

Objectives

Precursor Th17 lineage cells expressing CD161 are implicated in Rheumatoid Arthritis (RA) pathogenesis. CD4+CD161+ T-cells accumulate in RA joints and may acquire a non classical Th1 phenotype. The endogenous ligand for CD161 is lectin-like transcript 1 (LLT1). CD161/LLT1 ligation may co-stimulate T-cell IFN-γ production. We investigated the presence and identity of LLT1-expressing cells in RA synovial fluid (SF) and synovial tissue (ST). We also assessed levels of soluble LLT1 (sLLT1) in different phases of RA development.

Methods

Paired samples of peripheral blood mononuclear cells (MC) and SFMC (n=14), digested ST cells (n=4) and ST paraffin sections (n=6) from late-stage RA were analyzed for LLT1 expression by flow cytometry and immunohistochemistry. sLLT1 was measured using a sandwich ELISA. Sera and SF from late-stage RA (n=26), recently diagnosed RA patients (n=39), seropositive arthralgia patients (SAP, n=31), spondyloarthropathy patients (SpA, n=26) and healthy controls (HC, n=31) were assayed.

Results

In RA SF, LLT1 was expressed by a small proportion of monocytes. In RA ST, LLT1-expressing cells were detected in the lining, sublining layer and in areas with infiltrates. The LLT1 staining pattern overlapped with the CD68 staining pattern. FACS analysis of digested ST confirmed LLT1 expression by CD68+ cells. Elevated systemic sLLT1 was found in all patient groups.

Conclusions

In RA joints, LLT1 is expressed by cells of the monocyte/macrophage lineage. Serum levels of sLLT1 were increased in all patient groups (patients with early- and late-stage RA, seropositive arthralgia and spondyloarthropathy) when compared to healthy subjects.

Introduction

Human T lymphocytes expressing killer cell lectin-like receptor CD161 (NKR-P1A) have gained increased appreciation over the last decade. CD161+ T-cells were identified as precursor Th17 cells involved in chronic auto-inflammatory disorders. Specifically, CD161+ Th17-lineage cells were implicated in the pathogenesis of Crohn's disease [1], giant cell arteritis [2] and psoriasis [3] by accumulation and active IL-17 expression in the disease-affected sites. CD161+ cells can acquire a non classical Th1 phenotype, manifested by IFN-γ and T-bet expression, thought to be driven by IL-12 at the site of inflammation [4,5]. CD161+Th1 cells have been reported to accumulate in the synovial fluid (SF) of juvenile idiopathic arthritis (JIA) [5] and rheumatoid arthritis (RA) patients [6].

The sole endogenous ligand for CD161 is lectin-like transcript 1 (LLT1) [7,8]. Despite the growing evidence supporting a role of CD161+ T-cells in autoimmune pathology, no studies have yet addressed the expression of lectin-like transcript 1 in autoimmune conditions.

Human LLT1 is a product of the CLEC2D gene belonging to the C-type lectin domain family 2 (CLEC2) of the C-type lectin-like receptors (CTLR), which also includes CLEC2A (keratinocyte- associated C-type lectin, KACL), CLEC2B (activation- induced C-type lectin, AICL) and CLEC2C (CD69) [9,10]. Surface expressed LLT1 represents isoform 1 of the CLEC2D gene generated via alternative RNA splicing [11] and is the only protein isoform for which the ability to bind CD161 has been confirmed [12]. CLEC2D isoforms 2 and 4 are expressed as transmembrane proteins residing in the endoplasmic reticulum. Alignment of the predicted CLEC2D protein isoforms identified alternative splicing variant 5 as a putative soluble form of LLT1 [11]. Circulating leukocytes are characterized by low LLT1 expression at both the mRNA [11,13] and protein level [13]. LLT1 upregulation has been associated with the activation status of the cell. Surface-expressed LLT1 was detected in stimulated T-cells, B-cells and NK-cells. LLT1 was not found on circulating monocytes or immature monocyte-derived dendritic cells (DC) but became upregulated on TLR-activated mature monocyte-derived and plasmacytoid DC [13,14].

Previously, the LLT1-CD161 interaction was reported to co-stimulate T-cell effector functions and to enhance IFN-γ production (a feature associated with the Th1 phenotype) [7]. Our previous findings showing enrichment of CD4+CD161+ T-cells at the local inflammatory site and their local skewing towards the Th1 phenotype [6], prompted us to investigate whether LLT1 is upregulated in the pro-inflammatory environment of the disease-affected joints. We aimed to define which antigen presenting cells (APC) may participate in the crosstalk with CD161+ T-cells by analyzing LLT1 expression on different immune cell populations from synovial fluid and synovial tissue (ST) of late-stage RA patients. In addition, we hypothesized that LLT1 may be expressed as a soluble protein. We therefore assessed not only the presence of surface-expressed LLT1 but also its soluble form in the serum and synovial fluid from patients in different stages of disease. In addition, sera from seropositive arthralgia patients (SAP) who are at risk of developing RA [15,16] and patients with spondyloarthropathy (SpA), were included in this analysis.

Materials and methods

Study participants

Forty-four long-standing, treated RA patients; 54 recently diagnosed RA patients; 30 patients seropositive for anti-CCP and/or RF with (a history of) arthralgia (SAP), 26 patients with spondyloarthropathy (SpA) and 31 healthy controls were included in the present study (Table 1). Absence of arthritis in SAP was confirmed by physical examination of 44 joints by a trained senior rheumatologist (EB). SAP were treated with various non-steroidal anti-inflammatory drugs (NSAIDs) only. Fifty-four patients included in the early RA group had their blood drawn at diagnosis before start of treatment with disease modifying anti-rheumatic drugs (DMARD). Long-standing RA patients received, DMARDs, biologicals, NSAIDs and glucocorticoids. Most SpA patients received NSAIDs and only few received DMARDs and/or glucocorticoids. HC volunteers were found among employees at the University Medical Center Groningen. All RA patients fulfilled 1987 or 2010 American College of Rheumatology (ACR) classification criteria for RA. Written informed consent was obtained from all participants for use of their samples in biomarker research. All procedures were in accordance with institutional guidelines and approved by the local ethics committees of the University Medical Center Groningen (UMCG) and Medical Center Leeuwarden (MCL) [6,17].

Mononuclear cells derived from peripheral blood (PBMC) and synovial fluid (SFMC) from 14 out of 44 long-standing RA patients were used for FACS analysis of LLT1 surface expression.

To study surface-expressed LLT1 at the site of inflammation, 6 synovial tissue biopsies obtained from hand, shoulder or knee joints from long-standing, treated RA patients who underwent joint replacement surgery or synovectomy were processed for immunohistochemistry. In addition, ST biopsies obtained from 4 late-stage RA patients undergoing hip (n=1) or knee (n=3) joint replacement surgery were enzymatically digested for cell isolation.

To assess the presence of soluble LLT1, paired serum and synovial fluid samples from 26 out of 44 long-standing RA as well as serum samples from 40 out of 54 early RA patients; all 30 SAP; 31 HC and 26 SpA patient sera were assayed.

Detection of LLT1-bearing leukocyte subsets in paired PBMC and SFMC.

Following thawing of cryopreserved PBMC and SFMC, cells were resuspended at a concentration of $1x10^6$ cells/100 µl in PBS with 10% heat-inactivated human AB serum (CTL-Europe GmbH, Bonn, Germany) to block surface Fc receptors. The cell suspension was incubated for 30 min at room temperature (RT) with the following mouse monoclonal anti-human antibodies: CD3 PerCP (Cat No 347344, BD Bioscience, Breda, The Netherlands), CD56 APC-H7 (Cat No 302216), CD16 AlexaFluor700 (Cat No 302026, BioLegend, San Diego, CA, USA), LLT1 APC (clone 402659, Cat No FAB3480A, R&D Systems, Abingdon, UK), CD14 eFluor 605NC (Cat No 93-0149, eBioscience, Vienna, Austria). Cells were analyzed using LSR II flow cytometer (BD Biosciences) and data analysis was performed with Kaluza® analysis software (Beckman Coulter, Woerden, the Netherlands).

Table 1. Clinical and demographical characteristics of the subjects included in the study.

	HC	SAP	Early RA	Late RA	SpA
N	31	30	54	44	26
Age [yrs]; mean (SD)	54.6 (7.5)	50.4 (14.5)	58.0 (13.2)	51.8 (11.8)	42.4 (13.4)
Gender; % female	67.7	66.7	77.8	65.9	53.9
CRP [mg/l]; median (range)	nd	5.0 (5.0-29.0)	14.5 (5.0-108.0)	26.0 (4.0-185.0)	9.0 (3.0-48.0)
ESR [mm/h]; median (range)	nd	12.0 (2.0-69.0)	28.0 (2.0-96.0)	33.0 (2.0-120.0)	14.0 (2.0-48.0)
DAS28; mean (SD)	na	na	4.9 (1.4)	4.9 (1.7)	na
RF; % positive (n)	nd	80.0 (24)	70.4 (38)	77.3 (34)	0.0 (0)
Anti-CCP; % positive (n)	nd	90.0 (27)	74.1 (40)	81.4 (35)	11.5 (3)
BASDAI; mean (SD)	na	na	na	na	5.0 (2.3)
ASDAS; mean (SD)	na	na	na	na	3.2 (1.1)

HC = healthy controls; SAP = seropositive arthralgia patients; RA = rheumatoid arthritis; SpA = spondyloarthropathy; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; DAS28 = disease activity score 28; RF = rheumatoid factor; anti-CCP = anti-cyclic citrullinated proteins antibodies; BASDAI = bath ankylosing spondylitis disease activity index; ASDAS = ankylosing spondylitis disease activity score; nd = not defined; na = not applicable

Immunohistochemical detection of LLT1 in rheumatoid synovial tissue

Mouse monoclonal anti-human CLEC2D antibody (clone 4C7, Cat No H00029121-M01, Abnova, Taipei City, Taiwan) was used for the detection of LLT1 in the synovial tissues (diluted 1:100). To further characterize LLT1- expressing cells, consecutive tissue sections were stained with the following antibodies: mouse monoclonal against human macrophage marker CD68 (IgG3, Cat No M0876, diluted 1:100), rabbit polyclonal against human T-cell marker CD3 (Cat No A0452,diluted 1:300), mouse monoclonal against human B-cell marker CD20cy (IgG2a, Cat No M0755, diluted 1:50, all from Dako, Glostrup, Denmark). Briefly, 5 μm paraffin sections were deparaffinized and rehydrated using the standard procedures, followed by microwave heating in Tris-EDTA buffer (pH 9.0) at 99°C for 30 min in order to retrieve the antigens. After cooling and washing in three fresh changes of phosphate-buffered saline (PBS) for 5 min each, blocking of tissue endogenous peroxidase was performed by incubation with 0.1% hydrogen peroxide in PBS for 30 min. Avidin/ Biotin blocking kit was used according to the manufacturer's protocol (Vector Labs, Burlingame, CA, USA). Non-specific antibody binding was blocked by incubating the sections with PBS containing 20% goat serum or 2.5% horse serum for 20 min. Following washing with PBS, tissue sections were incubated with the primary antibodies diluted in PBS with 1% bovine serum albumin (BSA; Sigma-Aldrich, Zwijndrecht, the Netherlands) for 60 min at room temperature, washed with PBS and the following secondary antibodies reagents were used: anti-mouse Ig- alkaline phosphatase and mouse anti-alkaline phosphatase (APAAP) detection kit (Dako); goat anti-mouse IgG3- horseradish peroxidase (HRP) antibody (diluted 1:50; SouthernBiotech, Birmingham, AL, USA); anti-rabbit Ig- Peroxidase detection kit (Vector Labs) or goat anti-mouse IgG2a-HRP antibody (diluted 1:50). DAB substrate- chromogen system (Dako) or Fast Red phosphate substrate (APAAP detection kit) were used according to the manufacturer's instructions.

Pictures of 3-5 different areas of each section were semi-quantitatively scored by three independent researchers (PC, JB, MH) with the number of positive cells ranked on a 4-point scale: 0 = none; 1 = <5%; 2 = 5-50%; 3 = >50%. Within each area lining layer, sublining layer, vessels and infiltrates were scored separately for the expression of all markers used for the immunohistochemical staining. The synovitis (Krenn) score was calculated as described elsewhere [18]. Briefly, the synovitis score was defined as the sum of the score of the lining layer enlargement (from 0 [1 layer of lining cells] to 3 [> 5 layers of lining cells]), the score of the synovial stroma cell density (from 0 [normal cellularity] to 3 [greatly enhanced cellularity]) and the score of the inflammatory infiltrate (from 0 [no inflammatory infiltrate] to 3 [dense band-like infiltrate or numerous follicle-like infiltrates]). Obtained Krenn score indicates absence of synovitis when 0-1, low-grade synovitis when 2-4 or high-grade synovitis when 5-9.

Synovial tissue digestion and analysis

Cells were isolated from ST biopsies, obtained from 4 late-stage RA patients as described [6]. Cells were resuspended in RPMI-1640 containing 10% fetal bovine serum (FBS; Lonza, Breda, the Netherlands), 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen until analysis. ST cells from all 4 subjects were thawed at the same time and were stained with Fixable Viability Stain 450 (BD Biosciences) according to the manufacturer's instructions. Following washing with PBS containing 0.5% BSA and 2 mM EDTA, cells were resuspended in the washing buffer with Fc receptors blocking reagent (Miltenyi Biotech) and stained with the following mouse monoclonal anti-human antibodies: LLT1 PE (clone 402659, Cat No FAB3480P, R&D Systems), CD3 eFluor 605NC (Cat No 93-0037), CD19 PE-Cyanine7 (Cat No 25-0199, both from eBioscience), CD161 APC (Cat No 130-092-678, Miltenyi Biotech) for 30 min. at RT. After fixation and permeabilization with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), cells were stained with mouse monoclonal anti-human CD68 FITC antibody (Cat No 11-0689, eBioscience) for 30 min. at RT, followed by washing with the permeabilization buffer (eBioscience). ST cells were analyzed using LSR II flow cytometer (BD Biosciences), and data analysis was performed with Kaluza® analysis software (Beckman Coulter).

Detection of soluble LLT1 using ELISA

Serum samples were used to determine levels of sLLT1 with anti-CLEC2D/OCIL/LLT1 sandwich ELISA (Cat No MBS936829, MyBioSource, San Diego, CA, USA) according to the manufacturer's instructions. In order to assess the impact of RF interference on the assay performance, we investigated whether RF is bound by the anti-LLT1 detection antibody of the ELISA kit. For that purpose 16 RF-positive serum samples (12/16 positive for IgA-RF [range 45-3000 U/ml], 9/16 positive for IgG-RF [range 106-32 U/ml], 13/16 positive for IgM-RF [range 372-26 U/ml] were used. Fc-free RF was obtained for 8 out of 16 serum samples by incubation for 22 h at 37°C with pepsin (Sigma-Aldrich), diluted in sodium acetate buffer (0.1 M, pH 3.6) to a final concentration of 0.67 mg/ml. RF-deprived and untreated serum samples

were incubated on a rabbit IgG-coated plate used for RF measurement (IBL International, Hamburg, Germany). After washing, anti-LLT1 detection antibody was added to the plate and the assay was continued according to the manufacturer's instructions. We observed no RF binding by the anti-LLT1 detection antibody (OD values <0.4). RF removal did not cause further reduction of the observed OD values (data not shown).

Statistical analysis

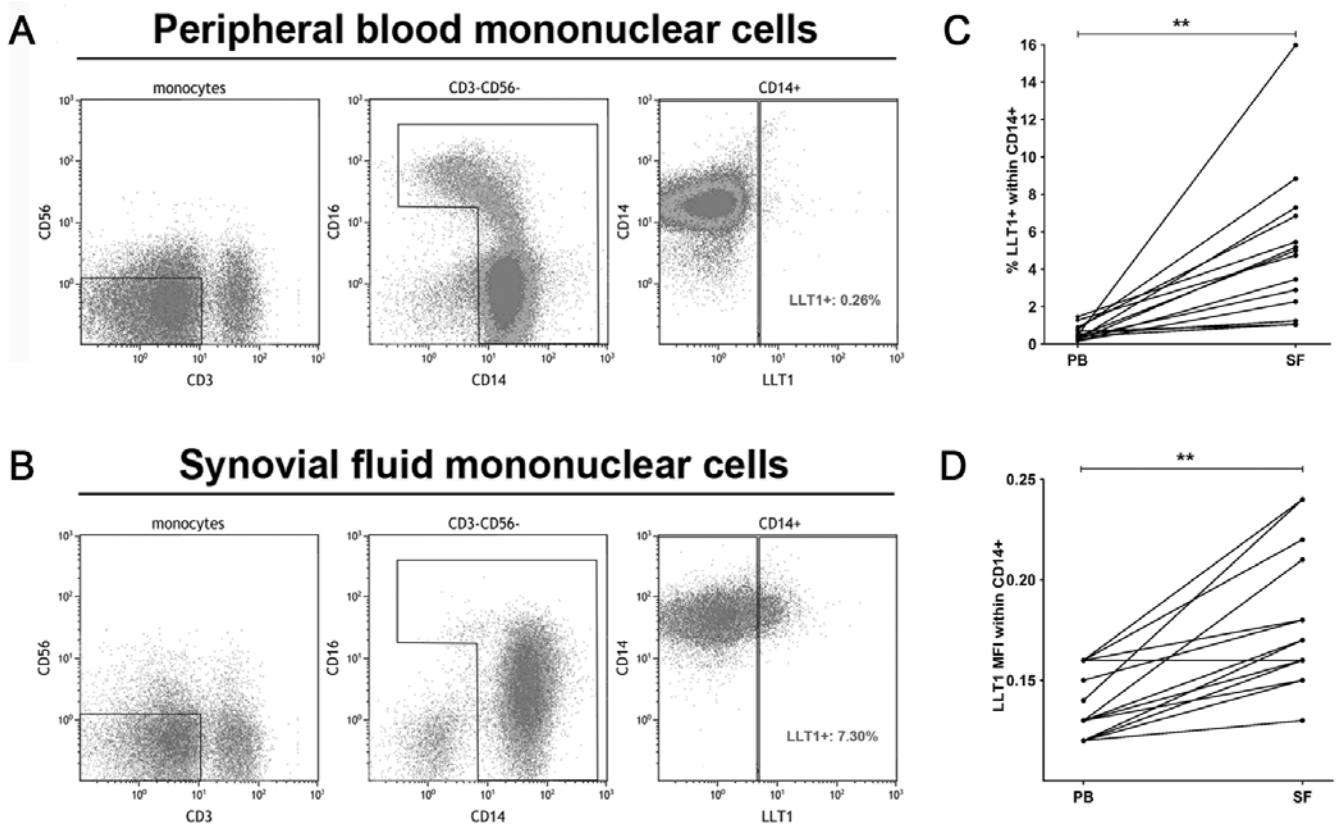
Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Normally distributed data were analyzed using unpaired t test. Non-normally distributed data were analyzed using Mann-Whitney 2-tailed test. Paired sample analysis was performed with Wilcoxon signed rank test. $P<0.05$ was considered statistically significant.

Results

Surface-expressed LLT1 detected on a proportion of synovial fluid monocytes

To investigate if LLT1 is upregulated in the pro-inflammatory environment of arthritic joints we first analyzed paired SFMC and PBMC from late-stage RA for LLT1 surface expression (Fig. 1A-D). Flow cytometric analysis detected LLT1 expression on a proportion of SF-derived monocytes whereas LLT1 was hardly detected on PB monocytes (median 5% [range 1.0-16.0%] vs 0.4%, [range 0,2-1,5%] of the CD14+ monocyte population) (Fig. 1A,B,C). LLT1 mean fluorescence intensity (MFI) was also significantly increased within the SF-derived monocytes compared to PB monocytes (Fig. 1D). In agreement with previous reports [11,13], we did not detect expression of LLT1 by circulating T-cells, B-cells, NK-cells, monocytes and granulocytes in PB samples of newly diagnosed RA or healthy controls (data not shown).

Figure 1. Surface-expressed LLT1 is found on SF monocytes.



Monocytes from A) peripheral blood and B) synovial fluid were gated based on forward and side scatter characteristics. After excluding CD3+ and CD56+ lymphocytes, monocytes were gated based on CD14 and CD16 expression. The frequency of LLT1+ cells was assessed within the total monocyte population. C) The frequency of LLT1+ monocytes and D) LLT1 MFI from paired samples of PB and SF (n=14). Mouse monoclonal anti-LLT1 antibody, clone 402659 (R&D Systems) was used.

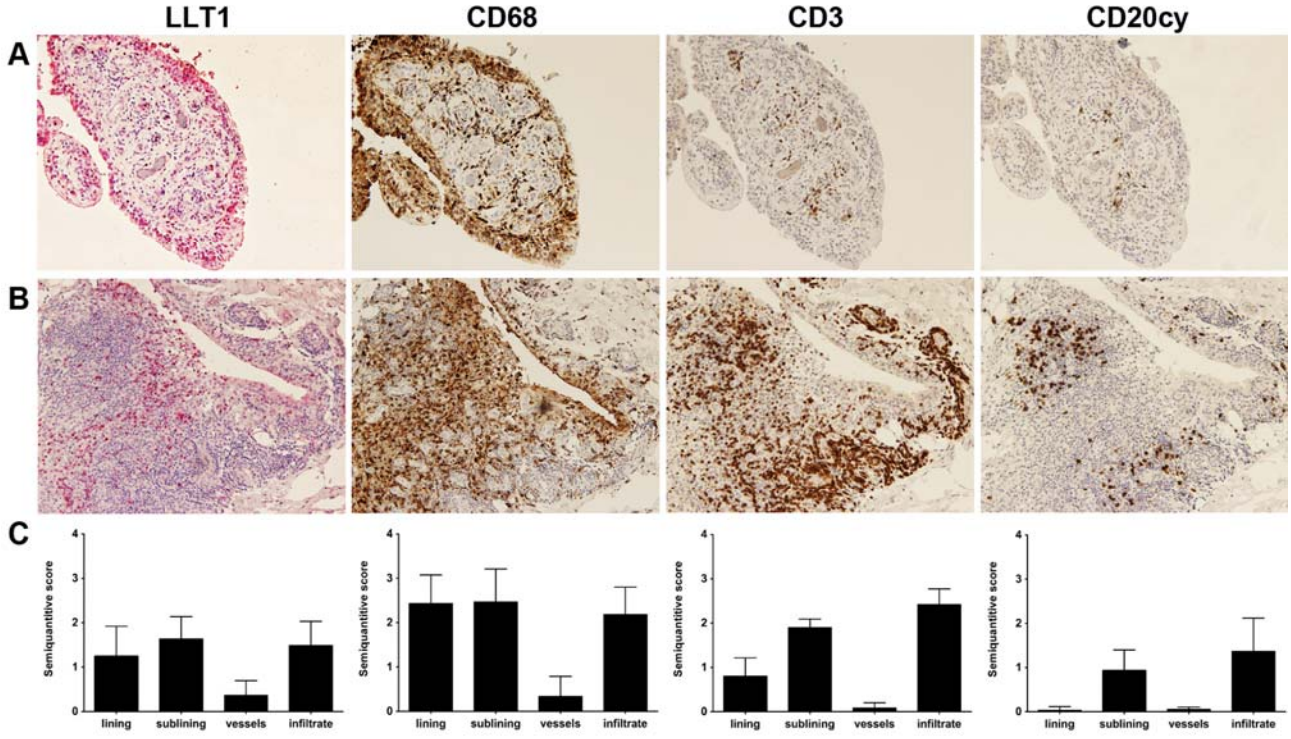
LLT1- expressing cells detected in RA synovial tissue

Following the detection of surface LLT1 by a proportion of SF monocytes, we next investigated the presence of LLT1-bearing cells in synovial tissue. To that end, synovial tissue specimens with low- to high-grade synovitis (median Krenn score of 5 [range 2-7]) [18] were obtained from six RA patients. Consecutive sections of synovial tissue were stained with antibodies against LLT1, CD68, CD3 and CD20cy (Fig. 2). LLT1 staining was detected in all six biopsies and was predominant in the lining layer which showed pathological enlargement in all 6 biopsies analyzed (≥1 point according to Krenn et al [18]). LLT1 staining was also found in the sublining and in transitional areas consisting of different cell types [19]. Small numbers of LLT1+ cells were observed in scattered lymphoid infiltrates and more dense perivascular infiltrates. These synovial membrane areas were defined based on CD3 (T-cells) and CD20cy (B-cells) staining. Analysis of the results of the semi-quantitative scoring showed that 3/6 tissue biopsies had low (median score 1, indicating <5% positive cells) and 2/6 biopsies had moderate (median score 2, indicating 5-50% positive cells) expression of LLT1 in the lining layer. LLT1 expression in the sublining layer was found to be low (median score 1) in 4/6 and moderate (median score 2) in 2/6 biopsies while the infiltrate showed low and moderate LLT1 expression in 3/6 and 2/6 biopsies, respectively. In addition, some LLT1 staining of blood vessels was observed. The LLT1 staining pattern

showed a marked overlap with CD68 staining. In all biopsies analyzed, CD68 expression was predominant in the lining and sublining layer and to a lesser extent in the lymphoid infiltrate areas.

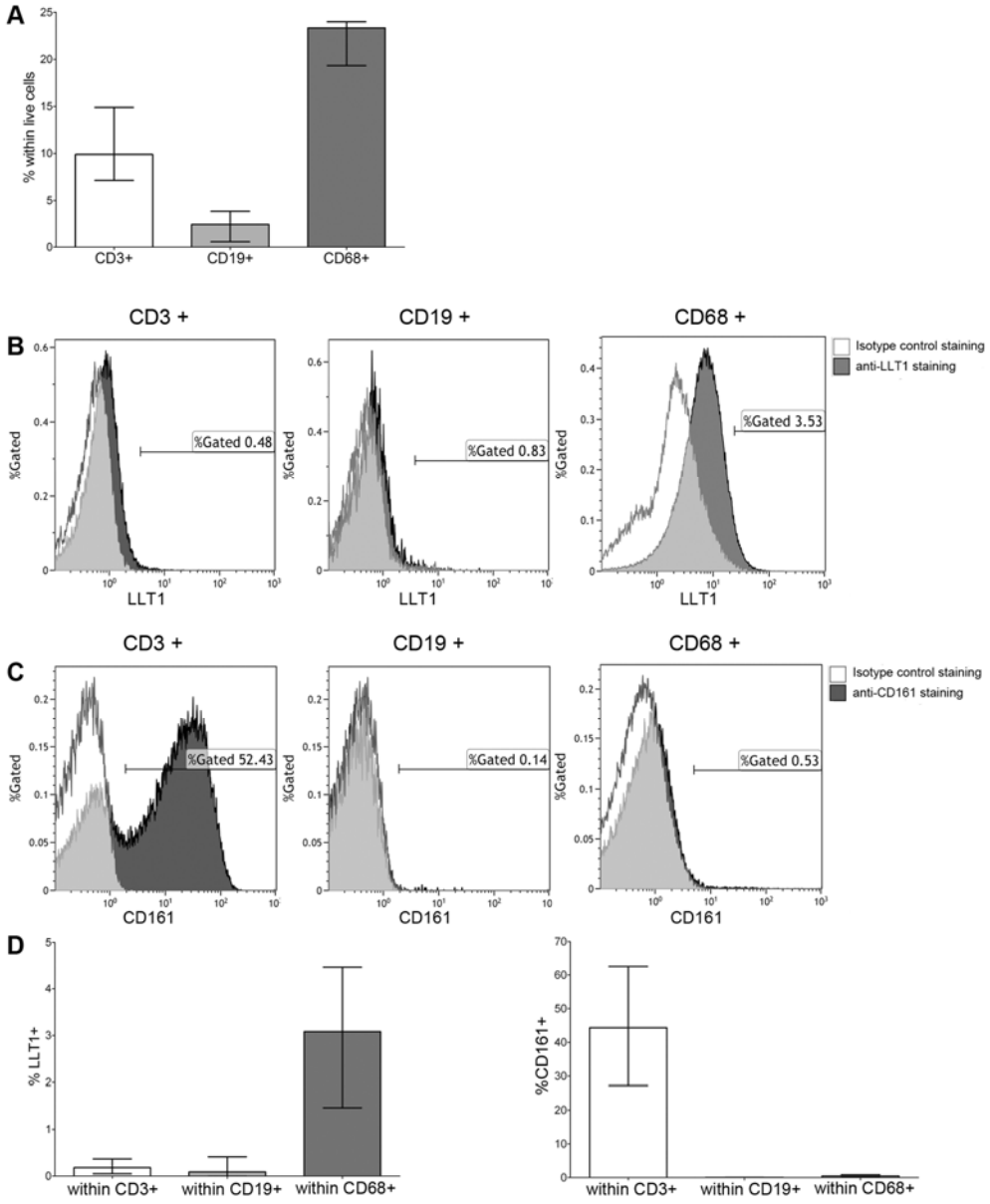
To further confirm LLT1 expression by synovial macrophages, we performed a flow cytometric analysis of LLT1 expression on cells derived from digested RA synovial tissue biopsies (n=4). The digested ST cellular composition constituted mainly CD68+ cells, T-cells and few B-cells (Fig. 3A). LLT1 expression was upregulated by ST CD68+ macrophages as evidenced by a shift in LLT1 mean fluorescence intensity (MFI, Fig. 3B). In contrast, synovial tissue derived B- and T-cells were found to be LLT1 negative (Fig. 3B,D). As before, a high percentage of CD161+ T-cells were detected [6]. CD161 expression was not detected on B-cells or on CD68+ macrophages (Fig. 3C,D). Thus, we conclude that in the ST, LLT1 and CD161 are expressed *in trans* by CD68+ macrophages and CD4+ T-cells, respectively (Fig. 3D). This would allow potential crosstalk of LLT1-bearing APC with CD161+ T-cells.

Figure 2. Immunohistochemical detection of LLT1 expression in RA ST cells.



To study surface-expressed LLT1 at the site of inflammation, 6 synovial tissue biopsies obtained from hand, shoulder or knee joints from long-standing, treated RA patients who underwent joint replacement surgery or synovectomy were processed for immunohistochemistry. Representative pictures showing immunohistochemical staining of consecutive tissue slides from 2 late-stage RA patients stained with antibodies against LLT1, CD68, CD3 and CD20cy (A,B). Graphs in C depict the results of the semiquantitative scoring (mean + SD) performed by 3 independent researchers. Scoring of the staining of all the markers was performed according to a 4-point scale: 0 = no positive cells; 1 = <5% positive cells; 2 = 5-50% positive cells; 3 = >50% positive cells. Three to five different pictures of each slide section (n = 6 different sections per biopsy) were taken. In each picture the lining, sublining, lymphoid infiltrate area's (defined based on CD3 and CD20cy staining) and blood vessels were scored separately for the expression of LLT1, CD68, CD3 and CD20cy. Mouse monoclonal anti-LLT1, clone 4C7 (Abnova) was used.

Figure 3. Flow-cytometric detection of LLT1 expression in RA ST cells



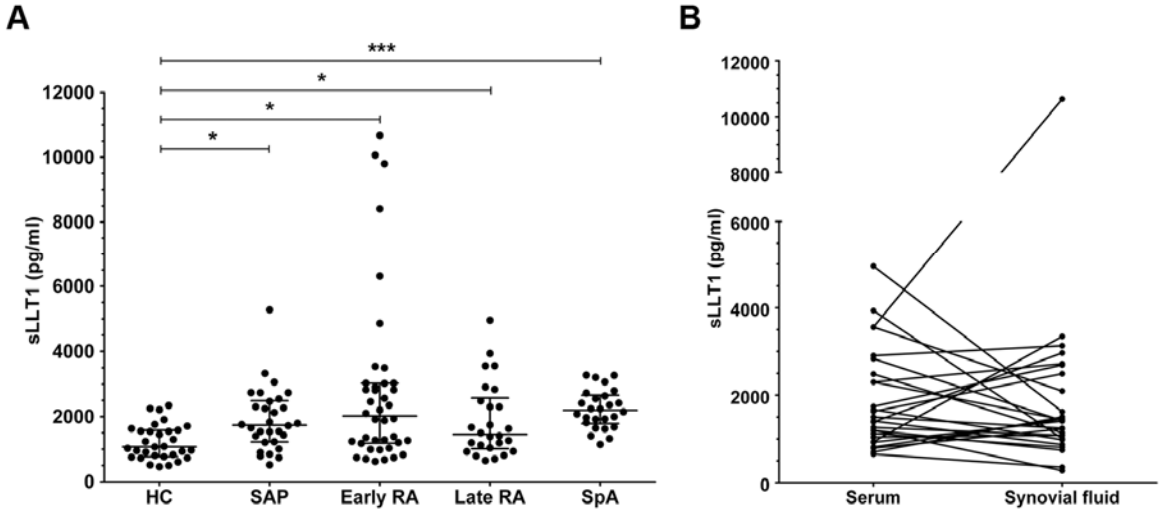
A) Percentages of CD3+ T-cells, CD19+ B-cells and CD68+ macrophages detected within the live cells gate of digested ST cells with flow cytometry. Briefly, necrotic cells were gated out based on the staining with the Fixable Viability Stain dye. Within the live cells gate lymphocytes and macrophages were gated based on FSC/SSC characteristics and CD68 expression, respectively. Within the lymphocyte gate T-cells and B-cells were gated based on CD3 and CD19 expression, respectively. Representative histogram overlays showing frequencies of B) LLT1+ and C) CD161+ cells within the populations of CD3+, CD19+ or CD68+ cells when compared to isotype control. D) Graphs show the percentages of LLT1 and CD161+ cells. Data from 4 independent donors were pooled. Bars represent the median value ± interquartile range. Mouse monoclonal anti-LLT1 antibody, clone 402659 (R&D Systems) was used.

Soluble LLT1 levels are elevated in sera from SAP, early and late-stage RA patients

sLLT1 was detected in the sera of SAP, early and late-stage RA patients using sandwich ELISA. Systemic LLT1 values ranged from 275 pg/ml to 10675 pg/ml. All patients groups, including SAP (mean sLLT1 level 1909 pg/ml [SD=971]), early RA (2813 pg/ml [2636]) and late-stage RA (1877 pg/ml [1137]) were characterized by significantly higher levels of sLLT1 compared to HC (1216 pg/ml [533]). To test if elevated sLLT1 is linked to the presence of autoantibodies and/or RF, we also tested sera from patients with RF-negative spondyloarthropathy (SpA, n=26). Here the levels of sLLT1 were also

significantly increased when compared to healthy controls (mean sLLT1 level 2225 pg/ml [SD=597]; Fig. 4A). Further, in late-stage RA, we did not detect an increase of sLLT1 in the synovial fluid when comparing paired SF and PB samples (Fig. 4B). The data suggest a systemic rather than a local elevation of sLLT1. Serum levels of LLT1 were not correlated with measures of general inflammation (CRP and ESR), RA disease activity (DAS28) or SpA disease activity (BASDAI and ASDAS; data not shown). We also did not observe a correlation between sLLT1 and disease duration (data not shown).

Figure 4. sLLT1 is increased in the serum of SAP, early and late-stage RA and SpA patients.



A) Sera from HC (n=31), SAP (n=31), early RA patients (n=39) and late RA patients (n=26) and SpA patients (n=26) were used to quantify the levels of soluble LLT1 using sandwich ELISA. Horizontal lines represent the mean value. Unpaired t test was used. B) Paired SF samples were used to compare the level of soluble LLT1 in PB and SF of long-standing RA (n=26; Wilcoxon matched pairs test). Statistical significance is indicated as * for $p < 0.05$, ** for $p < 0.001$, and *** for $p < 0.0001$. Rabbit polyclonal anti-LLT1 antibodies provided with a commercially available ELISA (MyBiosource) were used.

Discussion

This is the first study showing that surface-expressed LLT1 is present at the site of local inflammation in RA. The finding of LLT1 expression by cells of the monocyte/macrophage lineage in RA joints suggests potential crosstalk with CD161+ T-cells.

We aimed to identify which APCs express LLT1 at the level of the joint in RA. In late-stage RA, we investigated both the synovial fluid and the synovial tissue for the presence of LLT1 expressing cells. In SF, LLT1 expression was found in a small proportion of CD14+ monocytes. In RA synovial tissue, LLT1+ cells were found primarily in the lining layer enriched with macrophages [20] as evidenced by CD68 staining. It has been reported that the anti-LLT1 mouse antibody used for the tissue staining (clone 4C7) recognizes the CLEC2A isoform as well [11]. To confirm the detection of LLT1 in RA synovial tissue we performed a flow cytometric analysis of the digested ST using anti-LLT1 antibody (clone 402659) which is specific for LLT1. This antibody does not bind isoforms 2 and 4 of the CLEC2D gene nor other members of the CLEC2 family [11]. The combined data confirm that LLT1 is expressed by CD68+ ST macrophages.

We found LLT1 expression in RA joints to be confined to cells of the monocyte/macrophage lineage only. This may be explained by the notions that 1) LLT1 expression can be induced in B-cells and DCs following their *in vitro* stimulation with TLR ligands [13,14], 2) the expression of several TLR ligands [21-23] was previously found to be increased at RA inflammatory sites, 3) several pro-inflammatory cytokines (particularly Th1 cytokines such as IFN-γ) implicated in RA pathogenesis amplify the TLR-induced cellular activation, 4) T-cells [24], B-cells [25], monocytes [26] and neutrophils [27] in RA SF are characterized by an activated phenotype.

The presence of LLT1-bearing APC at the site of inflammation in RA may suggest their interaction with CD161+ Th17 lineage cells. Previous studies, including our own, have demonstrated the accumulation of CD4+CD161+ cells in RA joints where they show a non-classical Th1 phenotype [4,6]. Synovial fluid-derived IL-12 has been implicated in Th1 skewing [4]. CD161-triggering has been demonstrated to induce IFN-γ expression [7,13]. CD161 has a co-stimulatory role, thus the simultaneous engagement of TCR and CD161 is predicted to enhance T-cell function [7,28]. This suggests the contribution of the CD161-LLT1 co-stimulatory pathway to Th1 skewing at the level of RA joint. Our data demonstrate that the *in trans* expression of LLT1 and CD161 by CD68+ macrophages and CD4+ T-cells respectively, may allow for this intercellular (APC-T-cell) communication. Apart from their presence in the synovial lining and sublining, LLT1+ APC were also found in diffuse cellular infiltrates and perivascular lymphoid infiltrates where the presence and proximity of T- and B-lymphocytes were also demonstrated. Direct contact between T-cells and macrophages in RA synovium was demonstrated previously [29]. Technical limitations, however, prohibited a clear visualization of LLT1-CD161 interacting cells at the level of the ST. This was caused by incompatibility of the CD161 and LLT1 antibodies in different IHC protocols. More studies are thus required to formally prove that these cells interact locally and to prove that LLT1-CD161-mediated co-stimulation contributes to Th1 skewing at the level of the joint in RA.

Our studies also revealed LLT1 staining of the ST blood vessels. The data suggest a facilitating role for the LLT1-CD161 interaction in transendothelial migration of CD4+CD161+ T-cells to inflammatory sites.

In this report we show for the first time that soluble LLT1 can be detected in the peripheral blood and that sLLT1 levels are increased in autoinflammatory conditions when compared to the non-diseased state. Soluble LLT1 was found to be elevated in the sera of RA (irrespective of the disease stage), arthralgia patients who are at risk of developing RA [15,16] and patients with spondyloarthropathy. SpA is regarded as an autoinflammatory rather than an autoimmune rheumatic disease [30]. Similar to other proteins from the same C-type lectin-like domain 2 family, such as CLEC2A [31], AICL [32] and CD69 [33], LLT1 expression has been associated with the cellular activation status [11,13,34]. Its rapid upregulation by various cell types including T-cells, B-cells, NK-cells suggests that LLT1 may be an universal and early activation marker. In agreement with others [13] we did not observe surface LLT1 expression on ex vivo analyzed circulating immune cells. Increased levels of sLLT1 could indicate a prompt shedding following

cell activation. A similar phenomenon has been reported for soluble CD25 (sIL-2R) which is released upon cell activation [35]. sCD25 was found to be increased in the periphery of patients with chronic autoimmune diseases including RA [36]. A recent study provided evidence for LLT1 upregulation by non-hematopoietic cells (e.g. epithelial cells) in response to pro-inflammatory cytokines such as IL-1β, TNF-α or type I interferons [37].

More studies are required to assess if sLLT1 reflects ongoing (and past) systemic immune activation.

The demonstration of sLLT1 in patient sera raises the valid question if sLLT1 may have a putative functional role in the CD161-LLT1 interaction. Theoretically, sLLT1 may bind CD161 and as a consequence either block APC-T-cell interaction or signal via CD161. Although we cannot fully exclude a functional role for sLLT1 in RA, the available studies so far do not support a role for sLLT1 in CD161 binding. CLEC2D soluble isoforms failed to interact with CD161, leaving surface expressed LLT1 (isoform 1) as the sole ligand for this receptor [11].

Supplementary data

Detection of soluble CLEC2D using SDS-PAGE and Western Blot

Gel electrophoresis and Western Blot were performed in order to confirm the presence of soluble CLEC2D in human serum. Sera from 3 subjects were used: 2 (indicated as "s1" and "s2" in Suppl.Fig.1) had high levels of sLLT1 (10.065 and 10.675 pg/ml) and 1 (indicated as "s3" in Suppl.Fig.1) a low level of sLLT1 (466 pg/ml). In order to confirm the specific detection of CLEC2D, but not the other members of CLEC2 family (CLECA, CLEC2B, CLEC2C), the following recombinant proteins of CLEC2 family were used: CLEC2A (Thermo Scientific Pierce, Cat no PEP-1451 [synthetic peptide]); CLEC2B (Abnova, Cat no H00009976-P01 [recombinant protein]); CLEC2C (Abnova, Cat no H00000969-P01 [recombinant protein]), and CLEC2D (Abnova, Cat no H00029121-P01 [recombinant protein]). Briefly, Precision Plus Protein Kaleidoscope™ standard (Bio-Rad, Veenendaal, The Netherlands), recombinant proteins (final concentration 20 µg/mL), or serum samples (1:50) were loaded to Bio-Rad Criterion™ 12.5% gels (Bio-Rad) and were run under non-denaturating conditions (i.e. loading buffer without β-Mercapto-Ethanol or Dithiothreitol and without boiling) at 50 mA. After electrophoretic separation, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Amsterdam, The Netherlands) for 30 minutes at 100 V and blocked with Odyssey™ blocking buffer (LI-COR, Westburg, Leusden, The Netherlands) for 1 h.

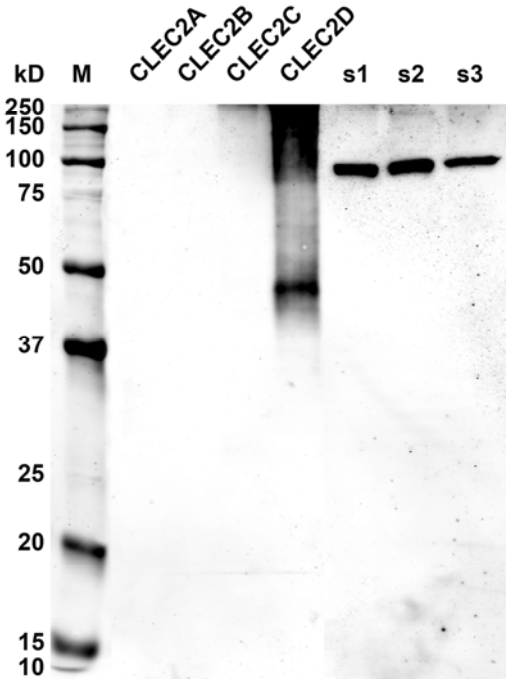
Following overnight incubation at 4°C with the Rabbit polyclonal detection antibody of the CLEC2D ELISA kit (1: 1000), the membrane was washed and incubated with the secondary antibody goat polyclonal anti-rabbit IgG IRDye680® (LI-COR)) for 1.5 h at room temperature and after washing scanned with Odyssey® CLx Western Blot Detection System (LI-COR).

Results

In order to confirm the specificity of the LLT1 ELISA and the detection of sLLT1 in human serum, we performed SDS-PAGE and Western blot. To this end we used the detection rabbit polyclonal anti-LLT1 antibody from the MyBiosource LLT1 ELISA kit. In addition, we used the mouse monoclonal anti-LLT1 antibody (clone 4C7). In Suppl. Fig. 1 the data show clear detection of recombinant CLEC2D but not the other recombinant CLEC2A, 2B and 2C proteins. The recombinant protein band was found to migrate at 46 kDa consistent with the molecular weight reported by the manufacturer. Mab 4C7 used in a parallel SDS-PAGE/WB gave a similar staining pattern, but of lower intensity (data not shown). Furthermore, in 3 different human sera we detected a clear positive band migrating just under 100 kDa under non-reducing conditions, thereby suggesting that sLLT1 may exist as a dimer in vivo.

The combined data show that the rabbit polyclonal anti-LLT1 employed in the sLLT1 detection kit is indeed specific for LLT1 and thus we may safely assume that it detects soluble LLT1 in human serum.

Supplementary Figure 1. Recombinant CLEC2D, but not other members of CLEC2 family, is detected in human serum using SDS-Page/Western blot.



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Chapter 7

Characteristics and potential involvement of CD70+ T-cells in seropositive arthralgia and in rheumatoid arthritis patients

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Work in progress

Abstract

Objectives

T-cells expressing CD70 are expanded in autoimmune diseases, including rheumatoid arthritis (RA). Upregulation of CD70 by CD4+ T-cells has been associated with a senescent phenotype on the one hand, while, on the other hand, CD70 has been reported as a specific marker for recently activated Th1 cells. We assessed if numbers of CD70+ T-cells (CD4+ and CD8+) were modulated by age, and characterized their phenotype and function by analysis of markers associated with activation, differentiation, cytokine production, proliferation and cellular senescence. Next, we assessed whether CD70+ T-cells are modulated in different phases of RA development.

Material and methods

Peripheral blood mononuclear cells (PBMC) were obtained from healthy controls (HC, n=19); seropositive arthralgia patients (SAP, n=28), representing subjects at risk of RA; newly diagnosed RA (n=23) and long-standing, treated RA patients (n=6). Also, synovial fluid mononuclear cells (SFMC) from late-stage RA were used. To characterize CD70+ T-cells, flow cytometric analysis using antibodies against CD45RO, CD27, CD28, CD69, HLA-DR, Ki-67, p16ink4a, T-bet, RORγt was performed. To investigate differences between T-cells from HC, SAP and early RA patients in their ability to regulate CD70 expression, PBMC were stimulated with PHA and cultured in vitro.

Results

Numbers of circulating CD70+ T-cells (CD4+ and CD8+) showed a strong positive correlation with age ($p<0.0001$). CD4+CD70+ T-cells were characterized by an increased expression of CD45RO, CD69, HLA-DR, Ki-67, p16ink4a and T-bet when compared to their CD70- counterparts. In RA, CD70+ T-cells were significantly increased at the level of the joint compared to the periphery. Frequencies of circulating CD70+ T-cells were not increased in PB of SAP and RA patients compared to HC. However, after in vitro stimulation, CD4+ T-cells from SAP and early RA showed an impaired ability to downregulate CD70 to baseline levels.

Conclusions

CD70+ T-cells (CD4+ and CD8+) increase with age and represent actively proliferating Th1-skewed effector cells rather than senescent T-cells. Accumulation of CD70+ T-cells at the level of the joint may suggest a role for these cells in augmentation of the local inflammatory process in RA. A delayed downregulation of CD70 by T-cells from SAP and RA may reduce the thresholds for activation of these cells and thereby contribute to the development of RA.

1. Introduction

Ample evidence suggests a central role of T lymphocytes in rheumatoid arthritis (RA) pathology (1, 2). The balance between T-cell activation and tolerance, crucial for immune homeostasis, is disrupted in RA and results in the dominance of a chronic immune effector response. The formation of such a response is dependent on the recognition of MHC- peptide complexes via specific T-cell receptor (TCR), as well as costimulatory signals derived from the same antigen presenting cells (APC). The most extensively studied costimulatory molecules, belonging to the B7/CD28 and TNF/TNFR families, include CD28 and CD27, respectively. Interaction between CD70 and its receptor CD27 is thought to be involved in the control of the tolerance-autoimmunity balance (3-5). Initiation of CD27-CD70 signaling is primarily regulated by the restricted expression of CD70. In contrast to CD27, constitutively present on the surface of T-cells, memory B-cells and NK-cells (6-8); CD70 is only transiently expressed on activated T-cells, B-cells, and dendritic cells (DCs) (9, 10).

Several studies demonstrated the involvement of excessive CD27-CD70 triggering in the pathogenesis of chronic infectious and autoimmune disorders. Increased numbers of CD70+ T-cells in systemic lupus erythematosus (SLE) have been implicated in the increased B-cell activation and antibody production (8, 11, 12). Similarly, blocking of the CD27-CD70 pathway in a mouse model of collagen-induced arthritis (CIA) led to a reduction of systemic autoantibody levels as well as decreased inflammation and joint destruction (13). Overexpression of CD70 on T-cells from rheumatoid arthritis (RA) patients has been demonstrated to provide a bystander co-stimulatory signal, derived not from APC, but third-party T-cells. This alteration, attributed to the defective T-cell ability to downregulate CD70 after stimulation, has been suggested to contribute to enhanced reactivity against low-affinity self-antigens (14).

Previously, CD70+ T-cells in RA were found to display a senescent phenotype (defined by their lack of CD28 expression) (14, 15). Accumulation of CD70+ T-cells in SLE has been associated with specific aspects of immune aging, i.e. the age-dependent decrease of DNA methylotransferase, involved in the suppression of CD70 gene transcription (5). We and others have demonstrated that CD70+ cells displayed an effector memory phenotype and an increased expansion potential (12, 16-19).

In this study, we first assessed if CD70+ T cells were modulated by age. We next characterized CD70+ T-cells in more detail by analysis of markers associated with activation, differentiation, cytokine production, proliferation and cellular senescence. Next, we assessed whether CD70+ T-cells home to the joint in late-stage RA. In order to investigate a role of CD70+ T-cells in RA development, the number of circulating CD70+ T-cells and the ability to regulate CD70 expression following in vitro stimulation were analyzed using peripheral blood mononuclear cells from seropositive arthralgia patients (SAP), who are at risk of developing RA (20-22) and from newly diagnosed RA patients.

2. Material and methods

2.1. Study participants

Venous blood collected in lithium heparin tubes was obtained from 19 healthy volunteers, 28 seropositive arthralgia patients (SAP), 23 recently diagnosed non-treated RA and 6 treated RA patients. Synovial fluid samples were collected from late-stage RA patients. Early RA patients had their blood drawn at time of diagnosis, before start of treatment with disease modifying anti-rheumatic drugs (DMARDs). Both SAP and early RA were treated with non-steroidal anti-inflammatory drugs (NSAIDs) only. Late-stage RA patients received methotrexate (3/6), prednisone (1/6), TNF-α inhibitors (3/6) and rituximab (1/6). All RA patients fulfilled the 1987 or 2010 American College of Rheumatology (ACR) classification criteria for RA. Absence of arthritis in SAP was confirmed by physical examination of 44 joints by a trained senior rheumatologist (EB). All participants gave their informed consent, and the study was approved by the local medical ethics committee (UMC Groningen, The Netherlands). Demographical and clinical characteristics of the included subjects are shown in Table 1.

Table 1. Demographical and clinical characteristics of the subjects included in the study.

	HC	SAP	Early RA	RA
N	19	28	23	6
Age [yrs]; mean (SD)	53.0 (7.5)	51.0 (14.6)	53.8 (12.6)	53.3 (5.9)
Gender; % female (n)	52.6 (10)	64.3 (18)	56.5 (13)	50.0 (3)
ACPA positive; % (n)	nd	92.9 (26)	73.9 (17)	100 (6)
RF positive; % (n)	10.5 (2)	85.7 (24)	73.9 (17)	66.7 (4)
CRP [mg/l]; median (range)	nd	5.0 (5.0-29.0)	12.0 (5.0-57.0)	5.0 (5.0-34.0)
ESR [mm/h]; median (range)	nd	11.5 (2.0-69.0)	22.0 (2.0-80.0)	15.0 (6.0-29.0)
TJC [n]; median (range)	na	1.0 (0.0-16.0)	7.0 (0.0-23.0)	2.0 (0.0-4.0)
SJC [n]; median (range)	na	0.0 (0.0-7.0)	6.0 (0.0-9.0)	2.0 (1.0-6.0)
DAS28; mean (SD)	na	na	4.6 (1.4)	3.3 (1.1)
Erosions; % (n)	na	na	4.3 (1)	66.7 (4)

HC = healthy controls; SAP = seropositive arthralgia patients; RA = rheumatoid arthritis; ACPA = anti-cyclic citrullinated proteins antibodies; RF = rheumatoid factor; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; TJC = tender joint count; SJC = swollen joint count; DAS28 = disease activity score 28; nd = not done; na = not applicable

2.2. Mononuclear cell isolation and staining

Mononuclear cells from peripheral blood (PBMC) and synovial fluid (SFMC) were isolated by Lymphoprep™ (Axis-Shield, Oslo, Norway) density gradient centrifugation. Isolated cells were resuspended in RPMI-1640 containing 10% fetal bovine serum (FBS; Lonza, Breda, the Netherlands) and 10% dimethyl sulfoxate (DMSO) on ice. The cell suspension was transferred in cryovials to Nalgene™ Cryo 1°C freezing containers (Thermo Scientific, Langenselbold, Germany) and placed in -80°C

overnight, followed by storage in liquid nitrogen until analysis. Thawed PBMC and SFMC were resuspended in PBS with 1% bovine serum albumin (BSA; Sigma-Aldrich, Zwijndrecht, The Netherlands) at a concentration 10^6 cells/100 μL and stained with the following anti-human antibodies: CD70 FITC, CD70 PE, CD45RO PE-Cy7, HLA-DR AF700, CD8 PerCP (BD Biosciences, Breda, the Netherlands), CD3 eF605NC, CD4 eF450, CD4 APC-eFluor780, CD27 PerCP-eFluor780, CD27 AF700 (eBioscience, Vienna, Austria), CD19 APC-Cy7, CD28 APC, CD38 AF700, CD69 PE-Cy7 (BioLegend, San Diego, CA, USA). For the intracellular markers detection, cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Antibodies against human Ki-67 PerCP-Cy5.5, p16ink4a PE (BD Biosciences) or T-bet PerCP-Cy5.5 and RORγt APC (eBioscience) were added to the cell suspensions in the permeabilization buffer (eBioscience) and incubated for 30 min at RT. Following washing with the permeabilization buffer (eBioscience), cells were analyzed using LSR II flow cytometer (BD Biosciences). Data analysis was performed with Kaluza® analysis software (Beckman Coulter, Woerden, The Netherlands).

2.3. PBMC in vitro stimulation and staining

Following thawing, PBMC were resuspended in RPMI-1640 with 10% FBS and 0.6% gentamycin (Life Technologies, Bleiswijk, The Netherlands), at a final concentration 10^6 cells/mL. Phytohaemagglutinin (PHA) was added at a final concentration 5 μg/mL. At day 4 and day 8 after start of the in vitro culture, cells were collected from wells and washed three times with medium. Next, cells were resuspended in RPMI-1640 with 10% FBS and 0.6% gentamycin at a final concentration 10^6 cells/mL. In vitro culture was continued in the presence of human recombinant IL-2 (Peprotech, London, UK) in a final concentration 100 U/mL. At day 13 after start of the in vitro culture, cells were washed and resuspended in fresh medium as previously and the culture was continued for the next 6 days without exogenous IL-2. Aliquots of PBMC collected at day 0, 4, 8, 13 and 18 during in vitro culture were used for analysis of the expression of surface and intracellular markers according to the procedure described above.

2.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). Correlations between the continuous variables were analyzed with Spearman's rank correlation coefficient. Paired samples analysis was performed with Wilcoxon signed rank test. Non-normally distributed independent samples were compared using Mann-Whitney 2-tailed test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Numbers of CD70+ T-cells increase with age

Both CD4+CD70+ and CD8+CD70+ T-cells correlated positively with age. This was observed for both the frequency and the absolute numbers of CD4+CD70+ ($p<0.0001$, $r=0.55$ and $p=0.004$, $r=0.41$, respectively) and CD8+CD70+ T-cells ($p<0.0001$, $r=0.53$ and $p=0.017$, $r=0.34$, respectively; Fig.1A,B).

3.2. CD70+ T-cells differ from CD70- T-cells in their phenotypes

We aimed to elucidate whether the observed increase of CD70+ T-cells with age is due to a gradual increase of senescent T-cells or due to expansion of the activated effector memory T-cell pool. CD70+ T-cells from healthy controls were analyzed for the expression of markers associated with a more differentiated phenotype such as surface CD28, CD27, CD45RO and expression of the intracellular senescence-associated p16ink4a. Also, markers associated with the activated cellular phenotype such as CD69, HLA-DR and the proliferation marker Ki-67, were assessed. To assess the putative function of age-associated CD70+ T-cells, we analyzed the expression of the transcription factors T-bet and RORγt, activators of IFN-γ and IL-17, respectively.

3.2.1. Markers of T-cell differentiation and senescence

Nearly all CD4+CD70+ T-cells expressed CD45RO, CD27 and CD28, indicating their memory, but not terminally differentiated phenotype (Fig. 1C). In contrast, CD4+CD70- T-cells showed significantly lower CD45RO expression (median 44.5% in CD4+CD70- vs. 89.8% in CD4+CD70+) and significantly higher CD27 expression (median 97.0% in CD4+CD70- vs. 91.1% in CD4+CD70+) suggesting a less differentiated phenotype. The percentages of CD28+ cells within both populations were similar. A small proportion (median 1.5%) of CD4+CD70+ T-cells expressed the senescence- associated p16ink4a which was found to be somewhat higher compared to CD4+CD70- T-cells (median 0.6%).

3.2.2. Markers of T-cell activation and proliferation

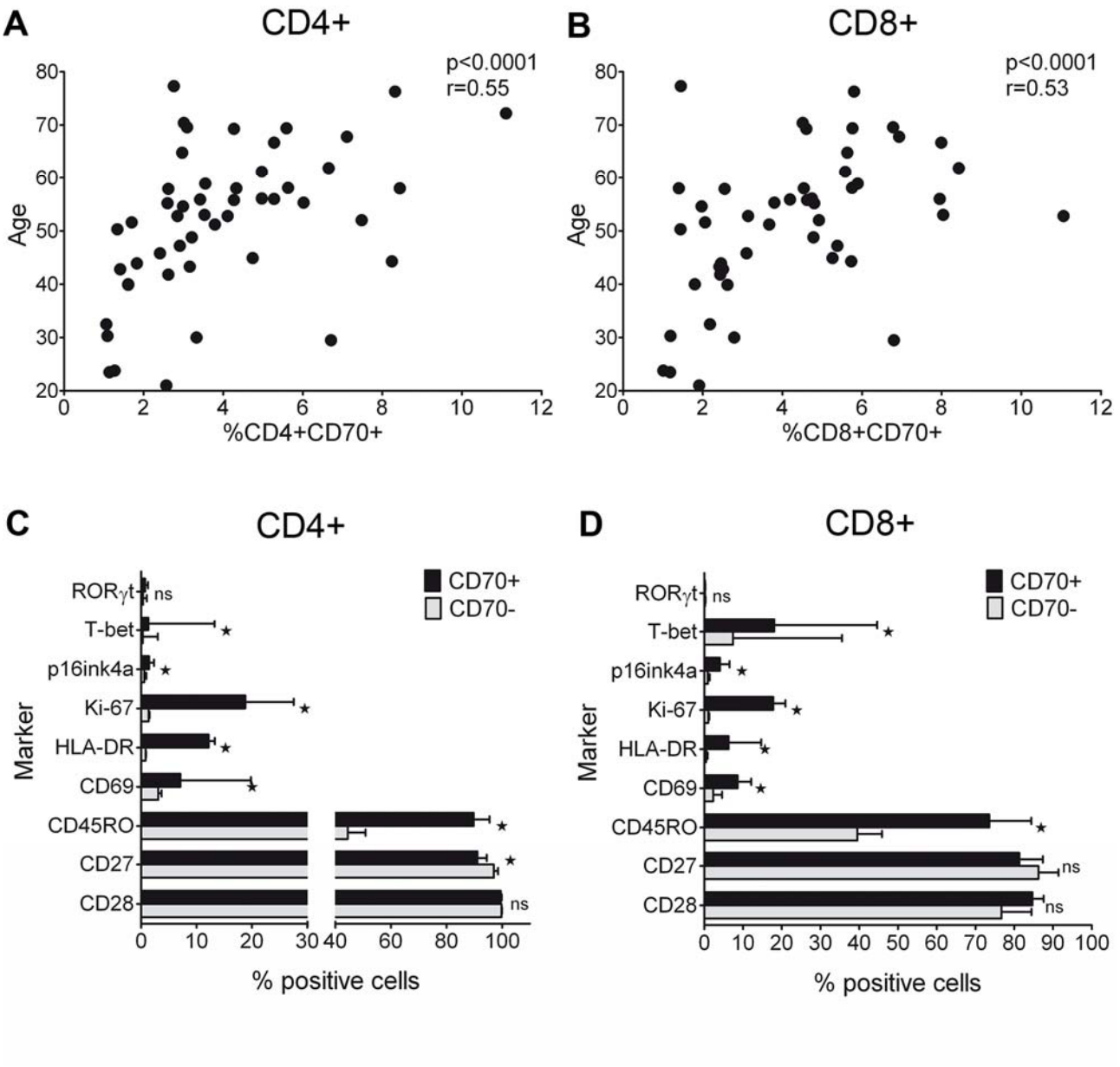
CD4+CD70+ T-cells were characterized by a significantly higher expression of the activation markers: CD69 (median 7.1% in CD4+CD70+ vs. 3.1% in CD4+CD70-) and HLA-DR (median 12.2% in CD4+CD70+ vs. 0.8% in CD4+CD70-).

The most pronounced difference between CD4+CD70+ and CD4+CD70- T-cells was the expression of Ki-67. Median 18.8% of CD4+CD70+ cells were Ki-67+ while only 1.4% of CD4+CD70- cells expressed Ki-67, suggesting that CD70+ T-cells in vivo show higher proliferation rates.

3.2.3. Transcription factors T-bet and RORγt

T-bet and RORγt were expressed by a minor fraction of both CD70+ and CD70- T-cells. However, CD4+CD70+ T-cells expressed T-bet at a significantly higher level than CD70- T-cells (median 1.3% in CD4+CD70+ vs. 0.3% in CD4+CD70-), while the expression of RORγt within both subsets was found to be similarly low (median 0.7% in CD4+CD70+ vs. 0.4% in CD4+CD70-). Similar observations for these markers were made for CD8+CD70+ and CD8+CD70- T-cells (Fig.1D).

Figure 1. CD70+ T-cells (both CD4 and CD8) increase with age and show increased expression of markers ccassociated with activation and proliferation.



A) CD4+CD70+ and B) CD8+CD70+ T-cells from 49 donors, including 10 HC, 28 SAP and 11 early RA were correlated with age (Spearman's rank correlation). Directly ex vivo isolated CD70+ and CD70- T-cells from the C) CD4+ and B) CD8+ T cell population obtained from HC were compared for surface expression of CD28, CD27, CD45RO, CD69, HLA-DR and intracellular expression of Ki-67, p16ink4a, T-bet and RORγt (Wilcoxon sign rank test).Bars and whiskers represent median and interquartile range. * indicates $p<0.05$.

3.3. Frequencies of peripheral blood CD4+CD70+ and CD8+CD70+ T-cells are not different in HC, SAP and RA patients

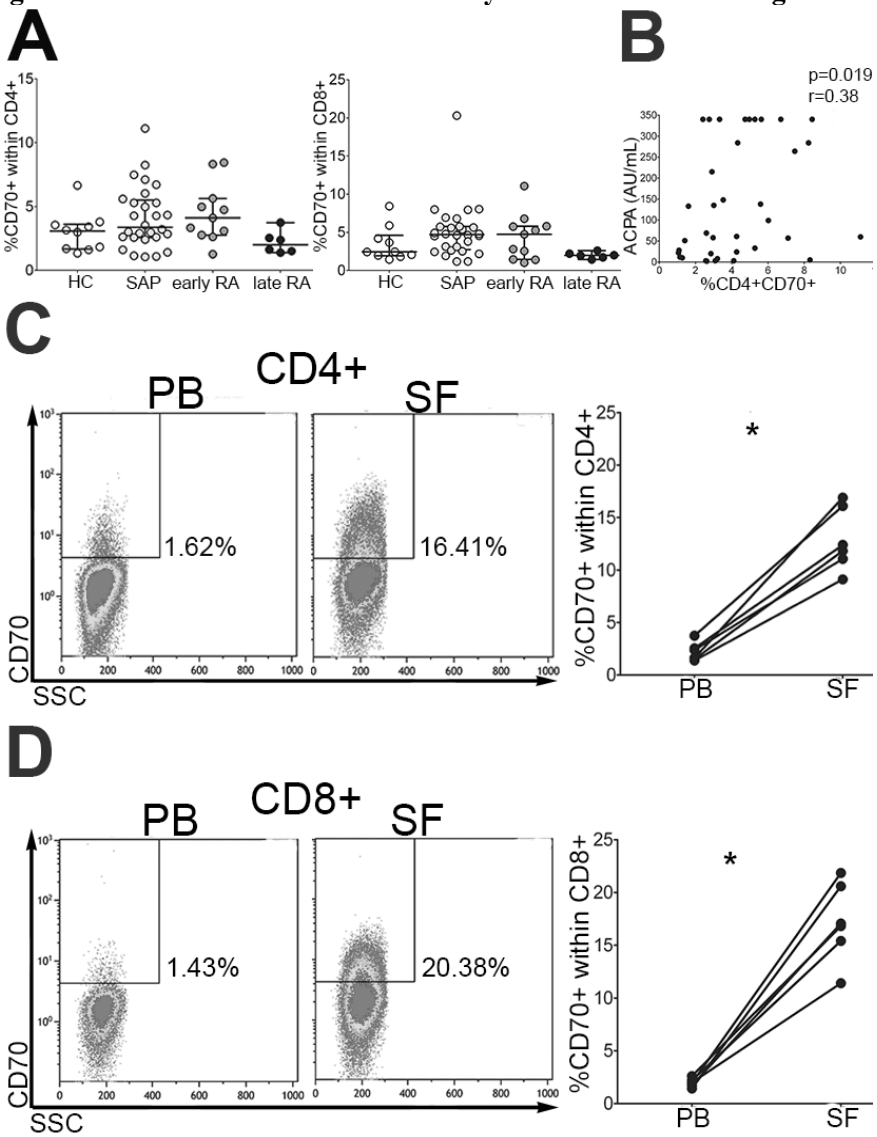
Increased frequencies of CD70+ T-cells have been described in various autoinflammatory conditions, including RA (14, 19). Within our cohorts of SAP, recently diagnosed RA and long-standing RA patients on treatment, the observed frequencies of CD4+CD70+ and CD8+CD70+ T-cells were not different from age-matched HC (Fig2A). Also, the phenotype of these cells did not differ from HCs (data not shown). Frequencies of CD70+ T-cells in SAP and RA did not correlate with clinical characteristics, such as CRP,

ESR, DAS28, TJC, SJC or RF level (data not shown). A weak positive correlation between the levels of ACPA and CD4+CD70+ T-cells was found (p=0.019, r=0.38; Fig.2B), in contrast to CD8+CD70+ T-cells (p=0.51, r=0.11). Interaction between CD70+ Th cells and CD27+ memory B-cells has been reported to induce plasma cell differentiation and subsequent immunoglobulin production (7, 8, 23, 24). We analyzed the correlation between the frequency of CD4+CD70+ T-cells and circulating plasma cells defined as $CD27^{high}CD38+$. No statistically significant correlation between these subsets was observed. Neither did the frequency of CD4+CD70+ T-cells correlate with the number of naïve (CcD27-CD38+), transitional (CD27-CD38+) or memory B-cells (CD27+) (data not shown).

3.4. CD70+ T-cells are significantly increased in RA synovial fluid

To investigate whether CD70+ T-cells may play a role at the site of local inflammation, their frequencies were analyzed in paired samples of PB and SF obtained from 6 long-standing RA patients. Both CD4+ and CD8+ T-cells expressing CD70 were found at significantly higher frequencies in SF (median 12.1% and 16.9%, respectively) compared to PB (median 2.0% for both CD4+ and CD8+) (Fig. 2C,D).

Figure 2. CD70+ T-cells accumulate in synovial fluid in late-stage RA.



Frequencies of CD4+CD70+ and CD8+CD70+ T-cells were assessed in PB of 10 HC, 28 SAP, 11 early RA and 6 late-stage RA patients (Mann-Whitney test). B) Correlation between the frequency of CD4+CD70+ T-cells and ACPA titers from 28 SAP and 11 early RA (Spearman's rank correlation). Frequencies of C) CD4+CD70+ and D) CD8+CD70+ T-cells in PB and SF of late-stage RA patients (n=6). Dot plots from 1 representative patient are shown. Graphs depict the pooled data from all late-stage RA patients (Wilcoxon sign rank test). * indicates $p<0.05$.

3.5. CD70 is transiently upregulated on the vast majority of T-cells following stimulation

Following characterization of ex vivo-derived CD70+ T-cells, we aimed to assess the expression kinetics of CD70 induction and the relationship between expression of CD70 and CD28, p16ink4a, CD69, HLA-DR or Ki-67. For that purpose, we performed an 18-day in vitro culture of PBMC. After 4 days in the presence of PHA, most of CD4+ T cells (median ~93%) were found to express CD70. Further culture in PHA-free medium led to a gradual downregulation of CD70 expression. Five (D8), ten (D13) and fifteen (D18) days after mitogen withdrawal, ~38%, ~13% and ~3% of CD4+ T-cells expressed CD70, respectively (Fig.3A). A similar induction and downmodulation of CD70 expression was seen in CD8+ T cells (data not shown). Percentages of CD70 expressing cells were higher than seen in CD4+ T-cells. At day 4, 8, 13 and 18 after start of culture, the median frequency of CD8+CD70+ T-cells was ~98%, ~54%, ~18% and ~3%, respectively (data not shown).

CD4+ T-cells expressing CD70 at day 4 were also positive for CD28. Since nearly all CD4+ T lymphocytes expressed CD28 at baseline, no further increase in the percentage of CD28 expressing cells was observed (Fig.3A). Despite the fact that CD70 upregulation was activation-dependent, only a fraction of CD70+ T-cells co-expressed other markers of activation, such as CD69 and HLA-DR (Fig. 3B,C). The frequency of CD70- T-cells expressing CD69 or HLA-DR during culture remained at a low level of ≤ 10%.

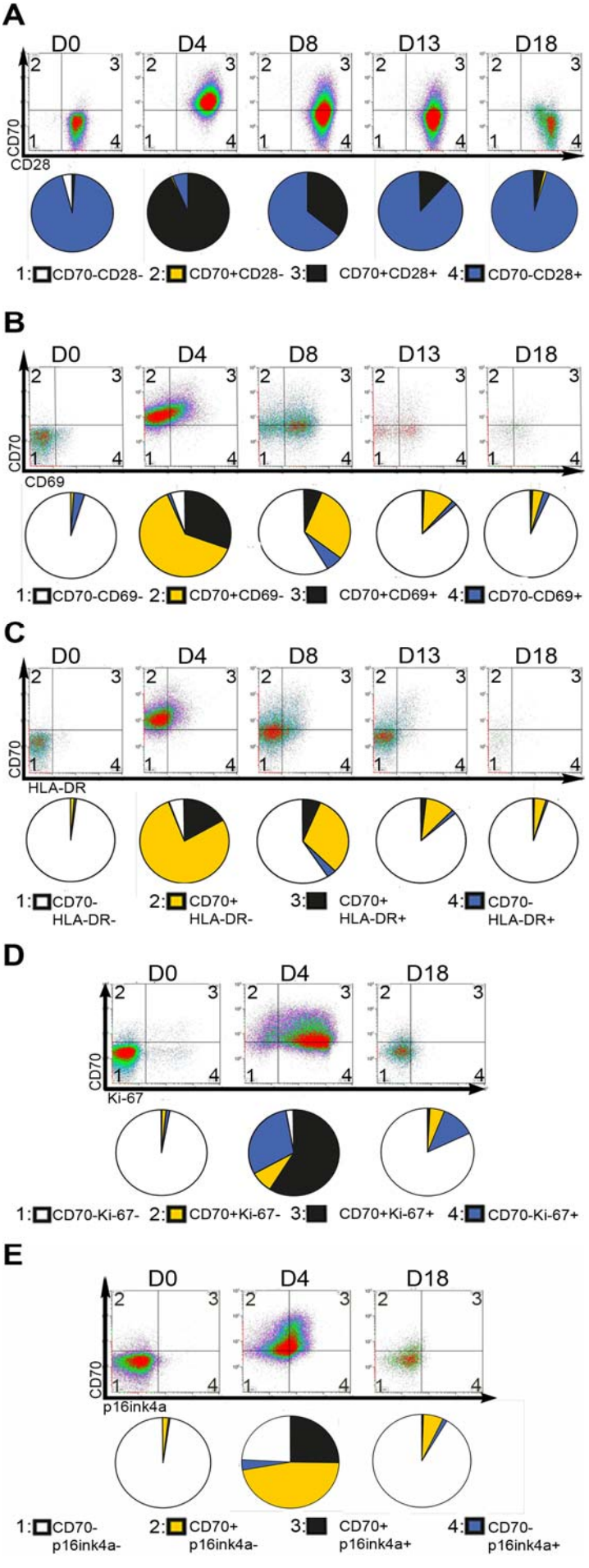
We observed that the induction of CD70 expression was associated with higher expression of Ki-67, indicative of cellular proliferation. Yet, not all proliferating cells showed CD70 expression. The percentage of CD70-Ki-67+ cells was found to be ~26% within the CD4+ T-cell population (Fig.3D). As demonstrated for CD69 and HLA-DR, also p16ink4a was found to be co-expressed by CD70+ cells following stimulation. The frequency of CD4+ expressing p16ink4a, but negative for CD70 was <4% (Fig. 3E). Similar expression kinetics were observed for CD8+ T-cells (data not shown).

In conclusion, in vitro activation of T-cells leads to co-expression of CD70 primarily with markers of T-cell activation and proliferation.

3.6. In vitro stimulated T-cells from SAP and early RA show delayed CD70 downregulation

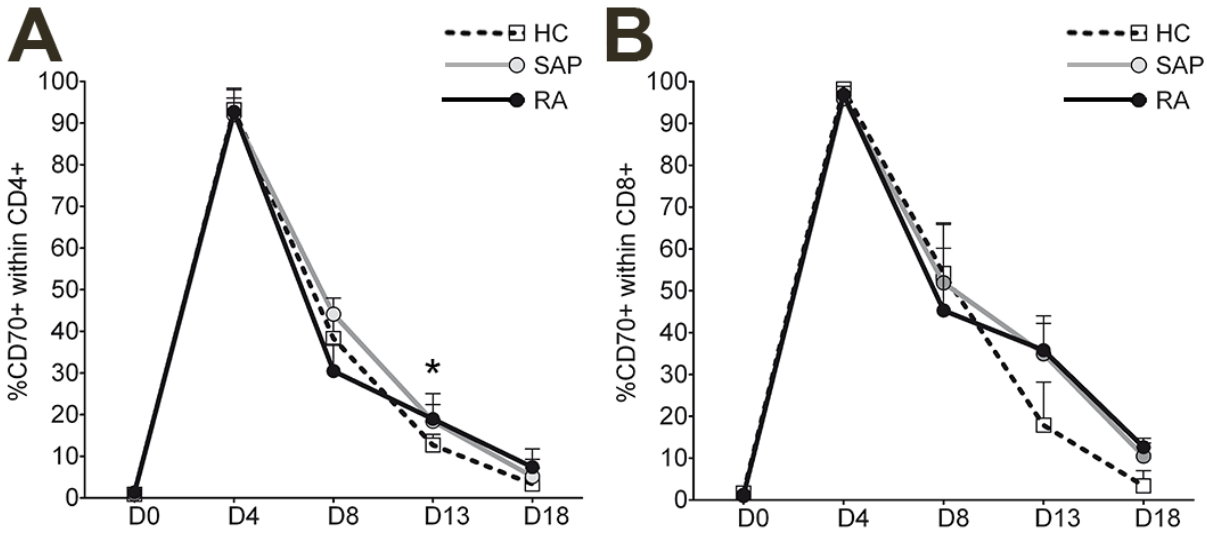
We next analyzed the dynamics of CD70 expression in the patient groups. During in vitro stimulation and subsequent gradual return to the resting phase, CD4+ T-cells from SAP and RA showed a decreased ability to downregulate CD70 compared to HC T-cells. Ten days after stimulus withdrawal (D13), a significantly higher frequency of SAP (18.4%, p=0.009) and RA T-cells (18.9%, p=0.037) expressed CD70 compared to HC (12.7%). Five days later (D18) SAP and RA CD4+ T-cells had downregulated CD70 expression to the level observed with CD4+ T-cells from HC (Fig.4A). CD8+ T-cells from SAP and RA showed a trend for a similarly defective downregulation of CD70, when compared to HC. However, the differences between SAP or RA and HC at day 13 (p=0.094, p=0.073, respectively) or at day 18 (p=0.054, p=0.097, respectively) did not reach statistical significance (Fig.4B).

Figure 3. Dynamics of CD70 expression and other markers of T cell activation and proliferation upon in vitro T-cell activation.



A-C) PBMC obtained form 7 HC were stimulated with PHA for 4 days followed by washing and culture in the presence of IL-2 alone for the next 10 days. After that, PBMC were maintained in culture with medium alone for the next 5 days. Co-expression of CD70 with A) CD28, B) CD69, C) HLA-DR was assessed at day 0, 4, 8, 13 and 18 of the culture. D), E) PBMC obtained from 6 HC were stimulated in the same way as described above. Co-expression of CD70 with D) Ki-67 or E) p16inka4 was assessed at day 0, 4 and 18 of in vitro culture. Representative dot plots from 1 HC are shown. Mean frequencies from the 4 quadrants within the dot plots are visualized by p ie charts (mean of n =7 or n =6 HC data).

Figure 4. Delayed CD70 downregulation by T-cells from SAP and RA.



Analysis of stimulation-induced CD70 expression regulation within A) CD4+ and B) CD8+ T-cells in long-term in vitro culture. PBMC obtained from 7 HC, 8 SAP and 7 early RA patients were ex vivo stimulated with PHA and CD70 expression by CD4+ and CD8+ T-cells was analyzed by flow cytometry (Mann-Whitney test). Symbols in the graphs represent median with interquartile range. * indicates $p<0.05$.

4. Discussion

Expansion of T lymphocytes expressing CD70 has been reported in various autoimmune conditions, including SLE (11, 12), Sjögren's syndrome (25), systemic sclerosis (26) and RA. In SLE, increased CD70 expression has been attributed to the age-related decline of T-cell DNA methyltransferase levels and subsequent hypomethylation of the CD70 promoter region (5, 15). In contrast, CD70 overexpression in RA has been attributed to the ill-defined defects in its downregulation (not associated with CD70 promoter hypomethylation) (14). In normal conditions, CD70 is transiently upregulated on activated T-lymphocytes, followed by a gradual downregulation (6, 10, 14, 16). In RA T-cells, reciprocal CD70 expression and acquisition of the senescent phenotype, manifested by CD28 loss, has been reported (14, 15). On the other hand, CD70+ T-cell expansion in SLE has been suggested to represent an activation-dependent rather than an age-dependent alteration (12). In our study, we did not observe increased frequencies of CD70+ T-cells in RA compared to age matched healthy controls. However, a significant positive correlation between the number of CD70+ T-cells and age prompted us to investigate the putative cause of the observed expansion. We aimed to establish whether CD70+ T-cells, increasing in elderly, represent aged T-cells bearing features of replicative senescence. Alternatively, age-associated systemic low grade inflammation (inflammaging) (27) may mediate bystander T-cell activation and accumulation of CD70+ effector T-cells.

Our data indicate that, in line with the second hypothesis, CD4+CD70+ T-cells show features of primed (CD45RO+, CD69+, HLA-DR+), actively proliferating (Ki-67+) cells, skewed toward the Th1 phenotype (T-bet+) when compared to CD4+CD70- T-cells. This is consistent with a study of Kawamura et al which demonstrated CD70 expression to be specific for Th1 but not Th2 cells (17).

Similar characteristics, including increased expression of T-bet within the CD8+CD70+ T-cell subset, suggest that CD70+ expression is associated with enhanced effector functions that could translate in vivo into increased IFN-γ production and cytotoxicity (28, 29) when compared to CD8+CD70- T-cells.

Similar to CD70+ T-cells characterized directly following ex vivo isolation, T-cells with stimulation-induced de novo CD70 expression were also positive for CD28 and Ki-67.

Our data confirm a previous observation by Lee et al on defective downregulation of stimulation-induced CD70 expression in RA (14). We here report that stimulation-induced CD70 downregulation is similarly delayed in SAP. This indicates a possible role for prolonged CD70 expression in RA development, as ~30% SAP has been shown to develop RA (20-22). Overexpression of CD70 on T-cells has been suggested to contribute to the induction of autoreactivity by virtue of lowering the activation thresholds of low-affinity T-cells (including those specific for self-antigens). It may also contribute to RA pathology by augmenting autoantibody production, as frequencies of CD4+CD70+ T-cells were found to correlate with ACPA titers. Indeed, CD27-CD70 interaction was shown to be involved in Th-mediated B-cell differentiation, plasma cell generation and immunoglobulin expression (7, 8, 23, 24). However, similar frequencies of CD4+CD70+ T-cells in RA patients and healthy controls suggest that additional RA-related factors are involved in autoantibody induction. Results of the study by Sammicheli et al. imply a pivotal role of IL-7 in this process (18). IL-7 was shown to induce CD70 expression on CD4+ T-cells which led to the subsequent CD70-mediated B-cell activation and immunoglobulin production. Furthermore, IL-7 was shown to induce expression of B cell activating factor (BAFF) which, in concert with anti-IgM F(ab′)2-fragments, has been demonstrated recently to stimulate ACPA production in vitro (30). Systemic levels of both IL-7 (31) and BAFF (32) have been shown to be markedly increased at the early stages of RA.

We confirmed accumulation of CD70+ T-cells in RA SF (33). CD70+ T cells may be involved in the recognition of (citrullinated) self or foreign peptides in the joints. IL-7, shown to induce CD70 expression, can also increase sensitivity of T-cells towards low-affinity self-antigens, including citrullinated peptides (34). We hypothesize that CD70+ T-cells preconditioned by the increased systemic IL-7, followed by triggering of TCR by self- peptide/MHC complexes in the local lymph node, would migrate preferentially towards the arthritic joint. Clearly, future studies are required to investigate this hypothesis

In conclusion, our study confirms the notion that CD70+ T-cells represent activated, actively proliferating Th1-skewed effector cells rather than terminally differentiated T-cells. Further studies should elucidate the role of CD70+ T-cell accumulation in normal aging. In early stages of RA (SAP and recently diagnosed RA), numbers of circulating CD70+ T-cells were not different from age-matched healthy subjects. However, defective downregulation of CD70 expression following stimulation in SAP and RA, as well as positive correlation with ACPA levels were observed. Further studies are required to understand the implications of these alterations for RA development.

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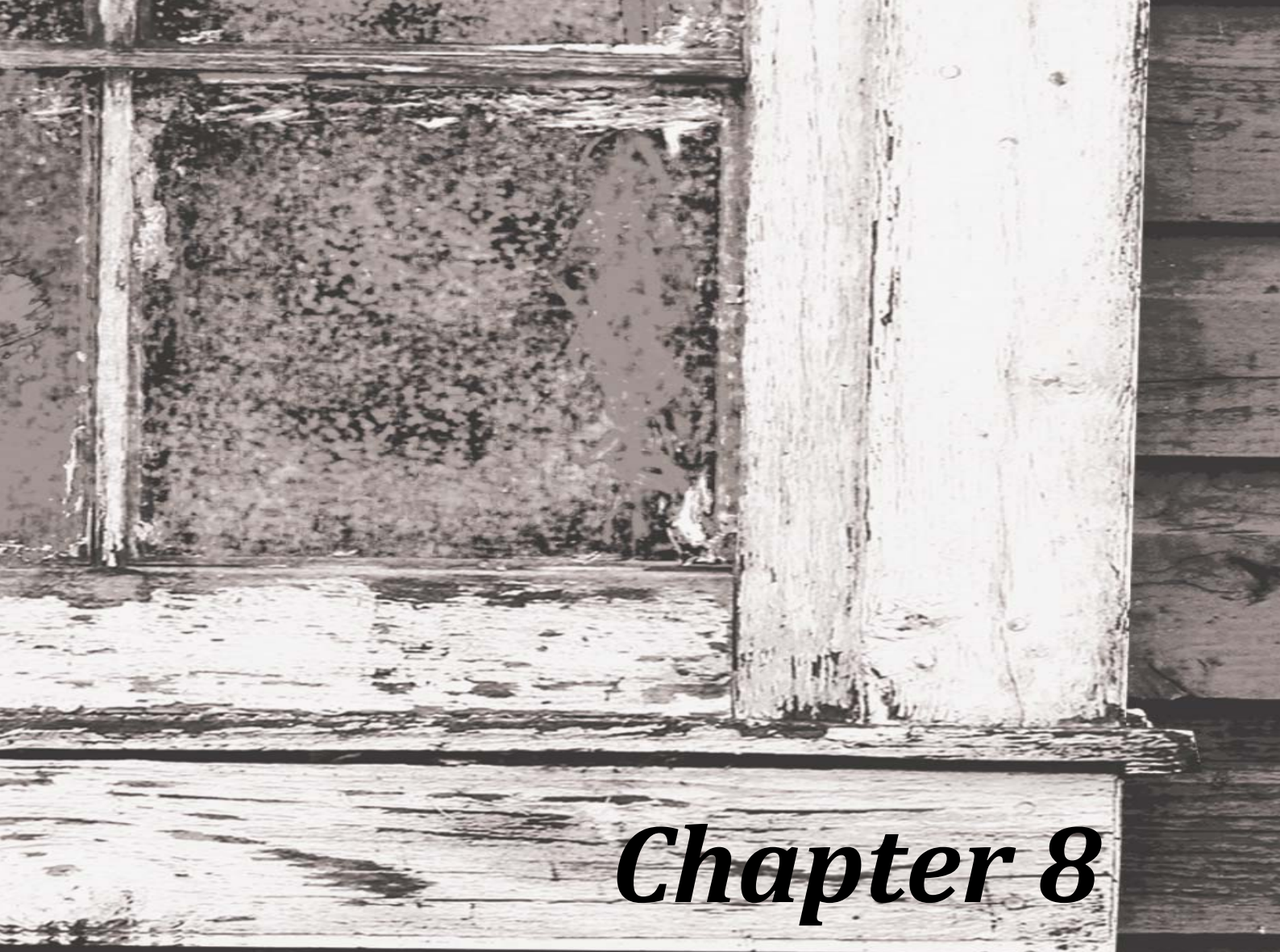

Chapter 8

Chronic autoimmune mediated inflammation, a senescent immune response to injury

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Abstract

The increasing prevalence of chronic autoimmune-mediated inflammatory diseases (AIMID) in ageing western societies implies a major challenge for the drug development industry. The current high medical need for more effective treatments is at least in part caused by our limited understanding of the mechanisms that drive chronic inflammation. Here we postulate a role for immunosenescence in the progression of acute to chronic inflammation via a dysregulated response to primary injury at the level of the damaged target organ. A corollary to this notion is that treatment of acute versus chronic phases of disease may require differential targeting strategies.

The immune system: for better or for worse

The primary function of the immune system is to protect the body against the detrimental effects of infection with viruses, bacteria or parasites, while at the same time damage to the infected tissues should be minimized. Although many threats come from the environment, also a healthy body carries many potential pathogens that need to be controlled, such as the bacteria that layer body surfaces (gut or skin) and the viruses in blood, lymphoid organs and bodily tissues. For its protective task the immune system is equipped with defense functions, which are in part already fully operational at birth (innate immunity) or which mature after birth in the daily engagement with environmental cues (adaptive immunity). While most people enjoy the benefits of immune protection for securing a healthy life, a substantial and steadily increasing proportion of the population, in 2000 ± 25% of the population in the USA; (http://mpkb.org/home/pathogenesis/epidemiology), experiences the hazardous consequences of unwanted and often detrimental immune activities. Examples of such conditions are allergy and autoimmunity, which are both driven by a dysregulated hyper reaction of the immune system. In the case of allergy the response is directed against environmental factors (e.g. pollen, food components, chemicals) and in the case of autoimmunity against components from body cells and tissues.

Autoimmune-mediated inflammatory disease (AIMID)

Inflammation results from the body response towards infection, irritation or tissue injury and is mediated by the immune system. Depending on severity, the clinical features of inflammation - pain, heat and swelling – can cause impairment of function. Inflammation is a complex biological process in organs and tissues aiming at the elimination of injurious factors and activation of the healing process. In a healthy individual, inflammation usually wanes when the insult has been eliminated and/or the injury has been healed. However, in certain clinical conditions inflammation does not wane but persists for prolonged periods of time. Chronic inflammation can occur for example when the immune reactions that drive the inflammation are directed against self-antigens present in or released from injured tissues.

The clinical course of AIMID is often characterized by an early phase dominated by inflammation with relatively more inflammation than tissue erosion, which can be treated with reasonable success using currently available immunotherapies, and a late phase where tissue degeneration is more pronounced than inflammation, for which an effective treatment is often lacking.

The lack of effective treatments for the chronic phase of AIMID is due to our limited knowledge of the mechanisms that underlie chronic inflammation and the lack of valid animal models (1). The poor predictive value of current AIMID animal models is a major hurdle in the translation of new therapeutic principles from the laboratory bench to the hospital bed (2).

Chronic inflammation

In most AIMID types the triggering event(s) is (are) not known. However, the subsequent exacerbations and remissions of clinical symptoms are believed to be mediated by the immune system. The exposure of

genetically predisposed individuals to (an) environmental trigger(s), infections in particular, has been proposed as a likely ethiogenic event, inducing the activation of autoreactive T and B cells present in the normal repertoire. The activation of autoimmune cells alone is usually not sufficient for the induction of overt clinical symptoms of autoimmune disease. Often additional pathogenic events within the target organ need to occur as well, including the activation of local APC and tissue injury, leading to the release of self-antigens and danger signals, named damage-associated molecular patterns (DAMPS), such as mitochondrial DNA (3), stress proteins (hsp60, hsp70) (4)or nuclear factors (e.g. HMGB1) (5).

As chronic AIMID are most prevalent in elderly people, it is thought that also age-associated changes in the immune system or the target tissues of the autoimmune attack enhance the risk of chronic inflammation, although the exact underlying mechanisms are poorly understood (1). The latter assumption warrants the question which age-associated changes of immune function enhance the risk to develop chronic AIMID.

MS and its animal model EAE, examples of prototypical AIMID

The difficulty to translate pathogenic and therapeutic concepts from the laboratory to the clinic can be illustrated by the situation in multiple sclerosis (MS). MS is a complex autoimmune-driven inflammatory disease affecting the human central nervous system (CNS), comprising the brain and spinal cord. The autoimmune pathogenesis of MS is modeled in experimental autoimmune encephalomyelitis (EAE). EAE can be induced in a variety of animal species (mice, rats, guinea pigs, primates) by active immunization with CNS antigens, mostly derived from the myelin sheaths that enwrap axons forming an isolation layer that facilitates fast pulse conduction (6). Although the EAE model has been instrumental for the development of several immunomodulatory/anti-inflammatory therapies (7), it has also been criticized as being an unreliable preclinical model (8,9).

Based on the response to treatment with immune modulating anti-inflammatory therapies, two phases can be distinguished in the pathogenesis of MS (6). Acute inflammation in the early disease phase responds well to some immunomodulating anti-inflammatory treatment, whereas inflammation in the late-stage chronic phase usually responds much poorer to these treatments. A representative example may be the beneficial effect of interferon-β on inflammation within the CNS white matter in relapsing-remitting MS and in EAE models, as detected on magnetic resonance images, whereas it has only a poor, if any, effect on clinical progression (10).

This discrepancy raises important questions:

1. Are early-acute and late-stage chronic disease driven by different pathogenic mechanisms?
2. Which immune alterations accompany or are at the basis of the transition from acute to chronic disease?
3. Which genetic and/or environmental risk factors steer the acute to chronic phase transition?

4. Is there experimental evidence for the existence of different immunopathogenic mechanisms in acute and chronic AIMID, from the EAE model for example?

Pitfalls of experimental disease models

While considering the relevance of animal models for the research of human AIMID the famous quote of the statistician George Box should be kept in mind: *"All models are wrong, but some are useful"* (11). Indeed, there is no animal model that recapitulates the complexity of human AIMID. However, this does not imply that all animal models are useless, as they can be of great help for the modeling of certain pathogenic mechanisms.

Despite the limitations, animal models have an important role in the preclinical research into disease mechanisms and the development of new therapies. Over the years, the inbred/SPF laboratory mouse has become the most frequently used animal model in preclinical AIMID research. However, it becomes increasingly clear that the immunological gap between a 10-12 weeks old SPF-bred mouse from a genetically homogeneous (inbred) strain and the complex patient population is more challenging than previously perceived and that this contributes to the frustrating situation that many new therapeutic entities fail to reproduce promising effects observed in a disease model when they are tested in patients. Hence, the question arises what can be learned from models in species that are more closely related to humans. Again, we use MS and its animal model EAE as an example.

The choice for a suitable animal model should be guided by the risk factors that have a well-documented influence on MS:

1. **Genes**: All genetic association studies reveal that the strongest genetic influence on MS susceptibility is exerted by the major histocompatibility complex (MHC) (12). This polygenic and highly polymorphic genomic region encodes molecules involved in antigen presentation to CD8+ and CD4+ T cells (MHC class I and II region) as well as effector molecules and their receptors (class III region). While selecting an animal model for translational research into AIMID pathogenesis and therapy, close genetic resemblance with humans enhances the relevance of the model.
2. **Environment**: Environmental factors with a recognized influence on the initiation and progression of AIMID are infection and vitamin D (13). We will not discuss the mechanism of action and therapeutic perspectives of vitamin D here and like to refer to reviews elsewhere (14) (15). An important difference between humans and laboratory rodents is that the human immune system has been shaped by the day-to-day exposure to new and existing infections. Viruses causing lifelong opportunistic infections, such as herpes viruses (CMV, EBV), and the bacteria in our gut flora (microbiota) have a particularly important impact on the human immune system.

In both respects, outbred colonies of conventionally housed non-human primates provide useful models for narrowing the gap between AIMID models in inbred/SPF rats and mice and the human population.

3. **Age**: The human immune system undergoes many changes associated with ageing of the body, which seem to be expressed more in the adaptive than the innate arm of the immune system. Documented changes include thymic involution, conversion of the CD4/CD8 ratio, decrease of the proportion of naïve T cells and progressive clonal expansion of terminally differentiated T cells lacking surface expression of CD28 ($CD28^{null}$) (16). The functional consequences of this immunosenescence process are reduced responses to vaccination, increased immune reactivity against autoantigens and an increased systemic inflammatory state (inflammaging). Although the exact mechanisms underlying these changes of immune function are incompletely understood, the chronic inflammatory state has been associated with clonally expanded, pro-inflammatory $CD28^{null}$ T cells (16). Here we postulate that the $CD28^{null}$ T cell subset has a direct pathogenic role in chronic inflammation and may thus be considered as a potential target of therapy.

Many of the age-related alterations observed in the non-human primate immune system resemble those in humans, including the oligoclonal expansion of $CD28^{null}$ T cells that mediate inflammaging at the expense of naïve T cells that can respond to new antigenic challenge (vaccination). Of note, CD28 loss is not observed in murine systems, thereby adding to the notion that mouse models do not fully capture immunosenescence features as observed in man (1).

These arguments plead for the non-human primate as inevitable preclinical model in drug development for AIMID. It should be noted that this is already common practice in transplantation immunology where non-human primates were proven to be better predictors for clinical success of new immunomodulatory treatments than rodents (17).

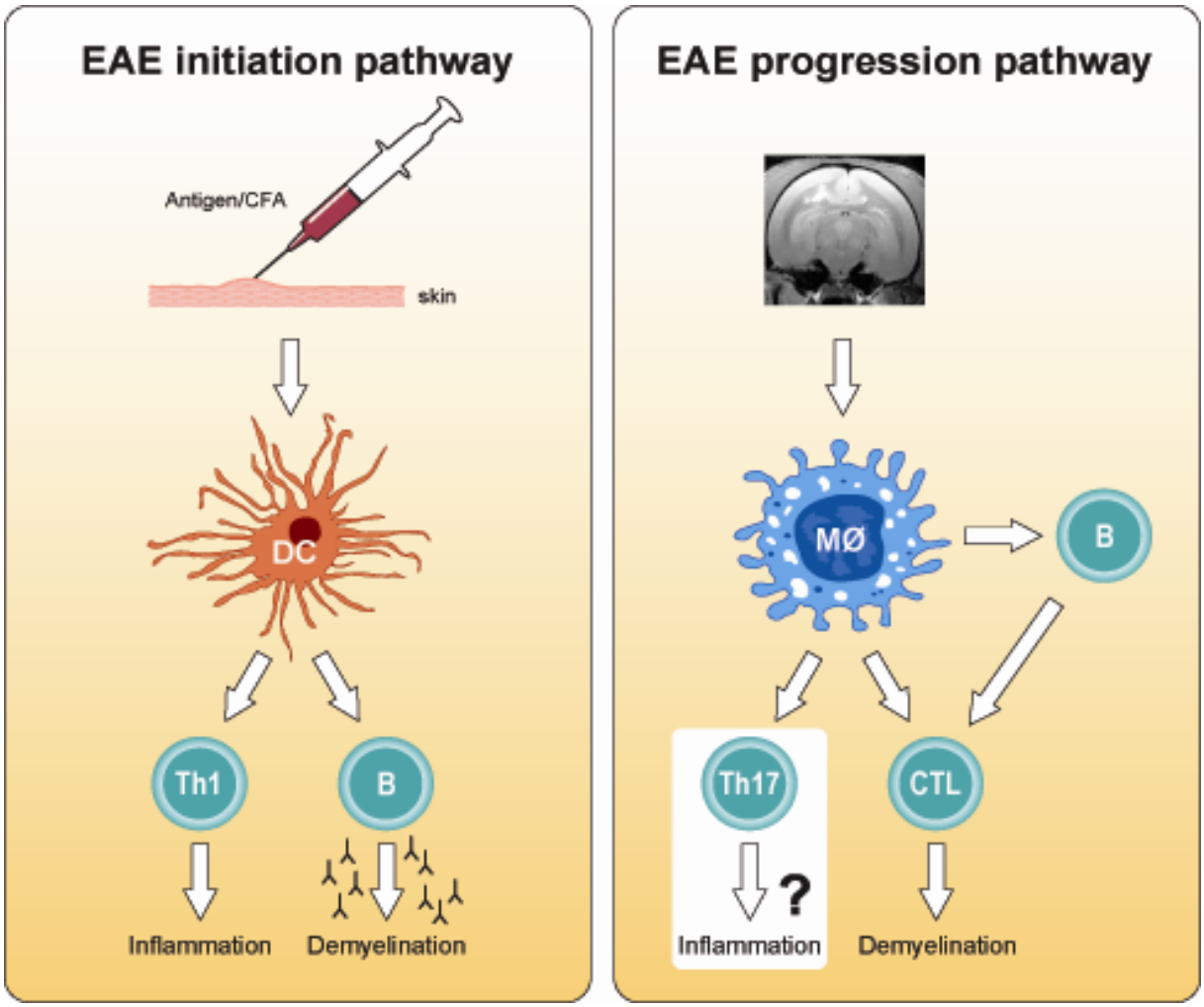
EAE in nonhuman primates

EAE has been induced in two macaque species, the rhesus (*Macaca mulatta*) and cynomolgus monkey (*Macaca fascicularis*) (for review: (18)). However, the ensuing disease is usually acute and seriously destructive, showing distant resemblance with the chronic progressive disease course in MS. The more recently developed EAE model in common marmosets (*Callithrix jacchus)* is much less severe and more heterogeneous in its clinical and pathological presentation than the rhesus monkey model, comprising cases with acute short-lasting disease and cases with chronic long-lasting disease (6).

Of the many CNS myelin components that can be used for EAE induction, the quantitatively minor, albeit specific, constituent myelin/oligodendrocyte glycoprotein (MOG) was identified as the most important autoantigen for induction of chronic disease in marmosets. This is best illustrated by the observation that marmosets immunized with MOG-deficient mouse myelin fail to develop chronic EAE, whereas their fraternal twin siblings do develop chronic disease (19). As an unglycosylated recombinant protein expressed in E. coli and formulated with the strong bacterial adjuvant CFA, MOG induces clinically evident EAE in almost 100% of marmosets from our outbred colony, but the disease course varies (20). Based on immune profiling data and the response to immunotherapy, we could conclude that acute

inflammation in the early disease phase and chronic inflammation in the late phase are driven by different immunopathogenic mechanisms (see Figure) (6).

Figure 1. Two pathways leading to EAE in marmosets.



Immunization of marmosets with rhMOG induces Th1 cells and the production of autoantibodies by B cells. The Th1 induce CNS inflammation, whereas binding of the autoantibodies to myelin sheaths induces demyelination via macrophage and complement dependent cytotoxicity. The initial autoimmune attack via this **classical pathway** elicits the release of autoantigens, which drain to cervical and lumbar lymph nodes, where (effector memory, EM) T cells are activated. These are characterized by high IL-17A production and specific cytotoxicity. It has not been elucidated whether these two activities are mediated by two different T cell types (Th17 and CTL) or that one T cell type (IL-17^{+}CTL) mediates both activities. The lack of CD28 expression and crossreaction with an immunodominant antigen of cytomegalovirus (major capsid protein; UL86) hints at the possibility that the EM cells may originate from the anti-viral memory repertoire. The secondary autoimmune attack via this **non-classical autoimmune pathway** results in pathological characteristics of progressive MS, i.e. microglia activation and demyelination of cortical grey matter. B cells are involved in the activation of the T cells mediating this progression pathway. Abbreviations: CFA = complete Freund's adjuvant; DC = dendritic cell; MΦ = macrophage or microglia cell

Early phase EAE: The early EAE phase in marmosets is driven by a canonical autoimmune mechanism that is strongly reminiscent of the EAE models in mice and rats (6). The inoculation of rhMOG/CFA into the dorsal skin elicits a uniform immunological event in all monkeys, namely the activation of T helper 1 cells specific for the epitope MOG24-36 together with autoantibodies against conformational MOG epitopes. The uniformity of the EAE initiation was explained by the fact that the MHC class II restriction element is a monomorphic MHC class II allele (*Caja-DRB1*W1201*) (21,22), which is ubiquitously

expressed in common marmosets (23). The synergistic action of Th1 cells and autoantibodies induces besides inflammation (22) and demyelination (24) also reversible axonal injury mainly localized in the white matter (25). Disease development in this early phase can be stopped by treatment with anti-human IL-12p40 antibody (26) or anti-CD40 antibody (27). The pathogenic mechanisms and response to therapy are very similar to those expressed in corresponding mouse EAE models.

Late phase EAE: The late EAE phase is driven by an unconventional autoimmune mechanism that has not yet been found in SPF rodent models but, as discussed elsewhere (6), a similar mechanism may be operational in MS. The variable onset of clinically evident EAE was found associated with the reactivation of CD3+CD4+CD8+CD56+CD27+CD28- effector memory cytotoxic T cells specific for MOG34-56 (28). The signature cytokine of this subset is IL-17A (29), but neutralization of IL-17A with a human-anti-human IL-17A antibody exerted little clinical effect (30). The specificity of the cytotoxic cells was defined at peptide 40-48 and the MHC restriction at the non-classical MHC class I^b allele Caja-E (31). In the Immuno Polymorphism (IPD)-MHC database (http://www.ebi.ac.uk/ipd/mhc/nhp/) only two Caja-E alleles have been published (*Caja-E*0301 and –E*0302*), which differ by a single nucleotide (triplet 138 ACG -> ACC). As the encoded amino acid is located outside the peptide-binding groove (position 107), the MHC class I molecules encoded by both alleles are likely functionally identical. The observation that the CD8+ T cells from monkeys sensitized against MOG34-56 cross react with peptide 981-1003 from the CMV major capsid protein (32) and that this response is MHC-E restricted point to a possible relation with a recently identified subset of HLA-E restricted NK-CTL in the human repertoire, which are engaged in the control of CMV infection (33,34). Based on this similarity we hypothesize that the CD3+CD4+CD8+CD56+CD27+CD28- effector memory T cells that have a core pathogenic role in the late EAE phase in marmosets and can be activated by immunization with MOG34-56 in IFA, originate from anti-CMV memory T cells present in the natural immune repertoire.

As discussed elsewhere (6), T cells are the key mediators in the EAE pathogenesis in marmosets but also B cells have a critical albeit different pathogenic contribution to early and late stage disease (see Figure). In the classical Th1-mediated pathway inducing early EAE the role of B cells is to produce autoantibodies that induce demyelination via cellular or complement–mediated cytotoxicity reactions (ADCC and CDC). In the non-classical CTL-mediated pathway inducing late stage EAE the main role of B cells is antigen presentation.

In summary, the similarities of the marmoset EAE model with MS include:

- the evidence for both an early acute and chronic phase of disease
- the almost immediate strong clinical effect of CD20+ B cells depletion (35,36),
- the involvement of $CD3+CD28^{null}$ T cells in chronic inflammation (37),
- the implication of CD3+CD4+CD56+ T cells in demyelination, by cytotoxic killing of oligodendrocytes (38,39).

Chronic inflammation, a response-to-injury paradigm

Surgical removal of CNS draining cervical (brain) and lumbar (spinal cord) lymph nodes impairs the chronic relapsing disease course in a Biozzi ABH mouse EAE model (40). The similar localization of myelin-laden APC within these lymph nodes during the course of EAE in mice and marmosets (41) and of MS in patients (42) supports an important role of these lymph nodes in the disease pathogenesis. Similar to marmosets, the chronic relapsing EAE course in Biozzi ABH mice is driven by autoimmunity against MOG (43). Based on these observations we have postulated a response-to-injury model for EAE and MS, which implies that MS is caused by a predisposed dysregulated immune reaction against antigens released from a damaged organ (44). The assumptions underlying this postulate are:

1. that primary injury inflicted in an organ causes release of self-antigens that either passively drain to lymph nodes as free molecules or are actively transported by phagocytic cells.
2. that T cells present in these draining lymph nodes exert a dysregulated hyper reaction to the released self-antigens. Conceptually, the combination of genetic and environmental factors predisposes an individual to a dysregulated autoimmune hyper reaction.

The cause of the primary injury can be diverse, including *i.* acute inflammation, as modeled in EAE, *ii)* a vascular problem, *iii)* tissue degeneration, as in neurodegenerative diseases, *iv)* virus infection. Actually, MS seems to share many pathological similarities with the vascular disease atherosclerosis (45). We like to state here that the autoreactive CD28 negative NK-CTL that were identified as core pathogenic factor in the late phase of marmoset EAE are an example of T cells capable to exert a dysregulated hyper reaction eliciting chronic AIMID.

$CD28^{null}$ T cells and (chronic) inflammation

One of the prominent features of immune aging is the oligoclonal expansion of CD4+ and especially of CD8+ T cells that lack expression of the co-stimulatory molecule CD28 (16). The expansion of $CD28^{null}$ subsets seems to be oligoclonal and partly the consequence of replicative stress due to recurrent exacerbations of (latent) cytomegalovirus (CMV) infection (46). $CD28^{null}$ cells seem to have lost proliferative potential but demonstrate enhanced survival (47). $CD28^{null}$ cell function is characterized by proinflammatory cytokine production and expression of perforin and granzyme B suggesting their cytotoxicity. Moreover, $CD28^{null}$ cells are relatively insensitive to suppression by regulatory T-cells (46). Many individuals with elevated numbers of $CD28^{null}$ cells in their circulation suffer from autoimmune disease. However, these cells rarely respond to disease-specific autoantigens, but rather to antigens from CMV or EBV (46) or to stress proteins, such as heat-shock protein (hsp) 60 (48). The central question therefore arises whether $CD28^{null}$ cells may be generic drivers of chronic inflammation in AIMID.

Is there a mechanistic explanation for a role of $CD28^{null}$ cells in the dysregulated immune reaction to injury? The $CD28^{null}$ effector memory T cell population acquires expression of several NK receptors

(both activating and inhibitory receptors). This may be a loss of function compensatory mechanism (49) mediated by reduced DNA methyl transferase activity, allowing the expression of methylation sensitive genes (50). Indeed, the methylation status of T cell derived DNA was recently shown to be age-dependent (51). The most frequently expressed NK receptor on $CD28^{null}$ cells is KIR2DL4 (CD158d), an activating receptor despite the presence of an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic tail (52). CD70 is another methylation sensitive gene that is expressed by $CD28^{null}$ T cells. CD70 expression may contribute to enhanced survival (53). Also, CD70 was found to lower T cell activation thresholds (54). Loss of CD28 associated with prominent expression of CD70 and *de novo* NK receptor expression infers that $CD28^{null}$ T cells are less dependent on cognate signaling (TCR/CD28) and thus sense their environment differently using receptor ligand (KIR-MHC class I) interactions characteristic of the innate immune system. Moreover, as CD28 loss is associated with increased production of pro-inflammatory cytokines and expression of cytotoxic effector molecules, CD28null T cells not only sense their environment differently but also are likely to respond differently and thus may be mediators of the dysregulated immune reaction to tissue injury.

In which AIMID has a possible pathogenic role of $CD28^{null}$ cells been documented?

Several chronic inflammatory diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus, Wegener's granulomatosis (GPA), atherosclerosis, inflammatory bowel disease and MS, are all characterized by expansions of $CD28^{null}$ T cells in the blood. Importantly, $CD28^{null}$ T cells have been detected at the site of pathology, suggesting their contribution to the disease process (MS and atherosclerosis). Here, we will briefly summarize the findings on $CD28^{null}$ T cells in RA, MS and in atherosclerosis.

RA: Weyand and Goronzy have reported on high relative percentages of $CD4^{+}CD28^{null}$ (up to 30-40% of CD4+ T cells) in patients with RA (55). Interestingly, premature accumulation of $CD28^{null}$ cells was found associated with carriage of the RA-associated HLA-DR4 subtypes. On the basis of these findings a novel disease hypothesis for RA was proposed (56,57). In subsequent studies, the expansion of $CD28^{null}$ cells in RA was confirmed in one third of patients and was linked to CMV seropositivity. Moreover, the expansion of $CD28^{null}$ cells was linked to the expression of the RA-associated HLA-DR4 subtypes in both RA and healthy controls (58), Also, according to a recent study anti-CMV seropositivity of RA patients, which is associated with increased frequencies of CD28null T cells and CMV-specific Th1 cells, was linked to a more severe disease course (59). Notably, $CD4^{+}CD28^{null}$ cells were most frequently found in patients with extra articular disease manifestations (e.g. vascular pathology).

It was previously suggested that $CD4^{+}$CD28null cells by virtue of CD161 expression home to the synovial tissue in RA (60). CD161+ cells were found in synovial tissue but CD28 expression was not assessed. Later, others failed to demonstrate the presence of $CD28^{null}$ at the site of pathology in RA (61). Indeed, our own observations, imply a role for CD4+CD161+ effector memory Th1 cells in RA synovitis.

MS: Expansions of pro-inflammatory CD4+CD28^{null} T cells in the peripheral blood of MS patients are less frequent than in RA but have been reported by several groups (47,62). As in RA, a correlation with CMV seropositivity was established. Importantly, CD4+CD28null cells were detected in the cerebrospinal fluid and in the inflammatory lesions in the brain. Mechanistically, the fractalkine – CX3CR1 pathway was found involved in the migration to the target tissue (63).

Compared to RA, relatively little mechanistic information is available on the pathogenic contribution of CD28^{null}CD161^{+} T cells in MS. In a genome-wide association study CD161 emerged as a candidate susceptibility locus in MS (64). In MS, upregulation of CD161 expression on IL-17^{+}CD8^{+} T-cells, has been reported. These cells were further characterized as CCR6^{+} EM cells (CD27$^{-/+}$CD45RA^{-}) with a pro-inflammatory profile and lack of perforin (37). The expression of CCR6 may enable these cells to immigrate non-inflamed CNS via a recently discovered route that circumvents the blood brain barrier i.e. via the choroid plexus where high expression of the CCR6 ligand CCL20 has been observed (65). CD4^{+} T-cells that express CD161 can differentiate into Th17 EM cells, a cell type with a presumed prominent pathogenic role in MS (66).

Atherosclerosis: Atherosclerotic vascular disease (atherosclerosis/ASVD) is a complex progressive inflammatory disease affecting the cardiovascular system. ASVD is an important co-morbid condition in patients with RA. The disease is pathologically characterized by inflammation and thickening of arterial walls. In the arterial walls both stable and unstable atherosclerotic plaques are found. Stable plaques, which usually cause limited or no clinical problems, mainly consist of extracellular matrix and smooth muscle cells. Unstable plaques also contain inflammatory cell infiltrates; these plaques can rupture and release thrombogenic material into the circulation causing the cardiovascular problems. Besides (lipid-laden) macrophages, T cells are consistently found in atherosclerotic lesions (67). In the early stages of atherosclerosis CD4 T cells (LDL specific) are held responsible for the initiation and progression of the disease, whereas in the advanced stage, a role for CD4^{+}CD28^{null} T cells in mediating atherosclerotic plaque instability was shown (68,69). CMV establishes persistent infection of arterial cell walls and CD4+ T cells specific for CMV contribute to atherosclerosis development (70). In certain clinical conditions, HIV-associated atherosclerosis for example, it could be shown that cardiovascular problems by CMV are mediated by CD4^{+}CX3CR1^{+} T cells (71). In view of the prior discussion it is tempting to speculate, but unproven, that CMV sustains the activation of pro-atherosclerotic CD4^{+}CD28^{null} T cells within the vessel wall.

Conclusions and implications for therapy of AIMID

Chronic AIMID are often characterized by prolonged and persistent inflammation and by new connective tissue formation. It may be a continuation of an acute form or a prolonged low-grade form. In some AIMID, such as RA and MS, chronic disease is pathologically associated with the formation of ectopic lymphoid structures, respectively within arthritic synovium (72) and MS meninges (73). It is suspected

that these newly formed sites of lymphoid neogenesis support the persistent production of pathogenic factors, such as autoantibodies and pro-inflammatory cytokines (74). According to an intriguing, but disputed new concept, EBV-infected B cells play a central role in the organization of these structures (75,76).

In this viewpoint we propose a mechanistic concept of chronic inflammation in AIMID. For this discussion we have used literature data, our own experimental data from the prototypical AIMID animal model EAE and our own studies on immune ageing markers in RA. The assumption that clinically unrelated AIMID may share immune mechanisms that drive chronic inflammation, is based on the outcome of genome-wide association studies (77).

In summary, the postulated concept comprises the following elements:

1. Chronic inflammation is driven by a dysregulated T cell hyper-response against antigens released from a primary injury in a body organ. The pathogenic factor inflicting the primary lesion can come from within the organ, such as a degenerative or vascular problem, or from outside the organ, such as an acute inflammation caused by an autoimmune attack .
2. Depending on the nature of the pathogenic event that causes the primary lesion, the released antigens can be self-antigens chemically modified by post-translational processes or can be *de novo* synthesized, such as stress proteins. We assume that immune tolerance against such antigens is weak or non-existent.
3. The hyper-reacting T cells originate from a repertoire of effector memory T cells induced by antecedent viral infections. The genetic background of the individual (e.g. MHC class I and II polymorphisms) determines whether these anti-viral T cells crossreact with look-alike epitopes within self-antigens.
4. Immunoageing and the replicative stress by recurrent exacerbation of latent infections (e.g. CMV, EBV) are associated with oligoclonal expansion of $CD28^{null}$ KIR expressing T cells, which can be activated by antigens released from an injured organ. These cells are less sensitive to the normal immune regulatory mechanism, such as Treg cells and adrenal hormones (corticosteroids). The paradox that the expanding $CD28^{null}$ T cells are mostly CD8+, but that CD8+ T cells do not readily respond to soluble self antigens has been addressed in the marmoset EAE model. Accumulating evidence suggests that EBV-infected CD20+ B cells contribute to late stage EAE by presentation to the cytotoxic T cells of MOG34-56 via non-classical MHC class I molecules from the HLA-E lineage (unpublished own observations).

What are the implications of the pathogenic concept discussed in this publication for therapy development? The concept postulates that inflammation in AMID is driven by the reactivation of pre-existing effector memory T cells present in the normal immune repertoire. The fact that these memory cells are already committed to their lineage may explain the poor translation of immunotherapies

intervening with the activation of naïve T cells or their tolerization from AIMID animal models to the corresponding human disease. The concept also proposes that age-associated changes in the immune system, in particular the expanding repertoire of $CD28^{null}$ T pro-inflammatory cells, may explain why therapies targeting mechanisms of acute inflammation in MS – such as corticosteroids, β-interferons or glatiramer acetate - loose efficacy with progression of the disease. Notably, (repeated) corticosteroid treatment /use may even enhance immunosenescence through further (steroid-induced) thymus involution and thus may inadvertently contribute to the accumulation of CD28null T cells in chronic diseases.

Data obtained from the marmoset EAE model demonstrate a similar central pathogenic role for B cells as in MS (78). The underlying mechanism is that the core pathogenic NK-CTL need antigen presentation by B cells for their activation (79). Intriguingly, B cells infected with EBV are particularly equipped for this task (own unpublished observation). These findings may not only give a mechanistic explanation for the clinical efficacy of anti-CD20 antibodies in MS and RA and for the association of these AIMID with EBV, but also warrant the search for treatments that specifically target the EBV-infected B cell.

Key messages:

- We postulate a generic paradigm for AIMID
- Acute autoimmune inflammation causes injury in an organ
- Chronic inflammation is a dysregulated T cell reaction against antigens released from injury
- T cells lacking CD28 ($CD28^{null}$) have a central role in the response to injury
- $CD28^{null}$ effector memory T cells are a hallmark of immune ageing

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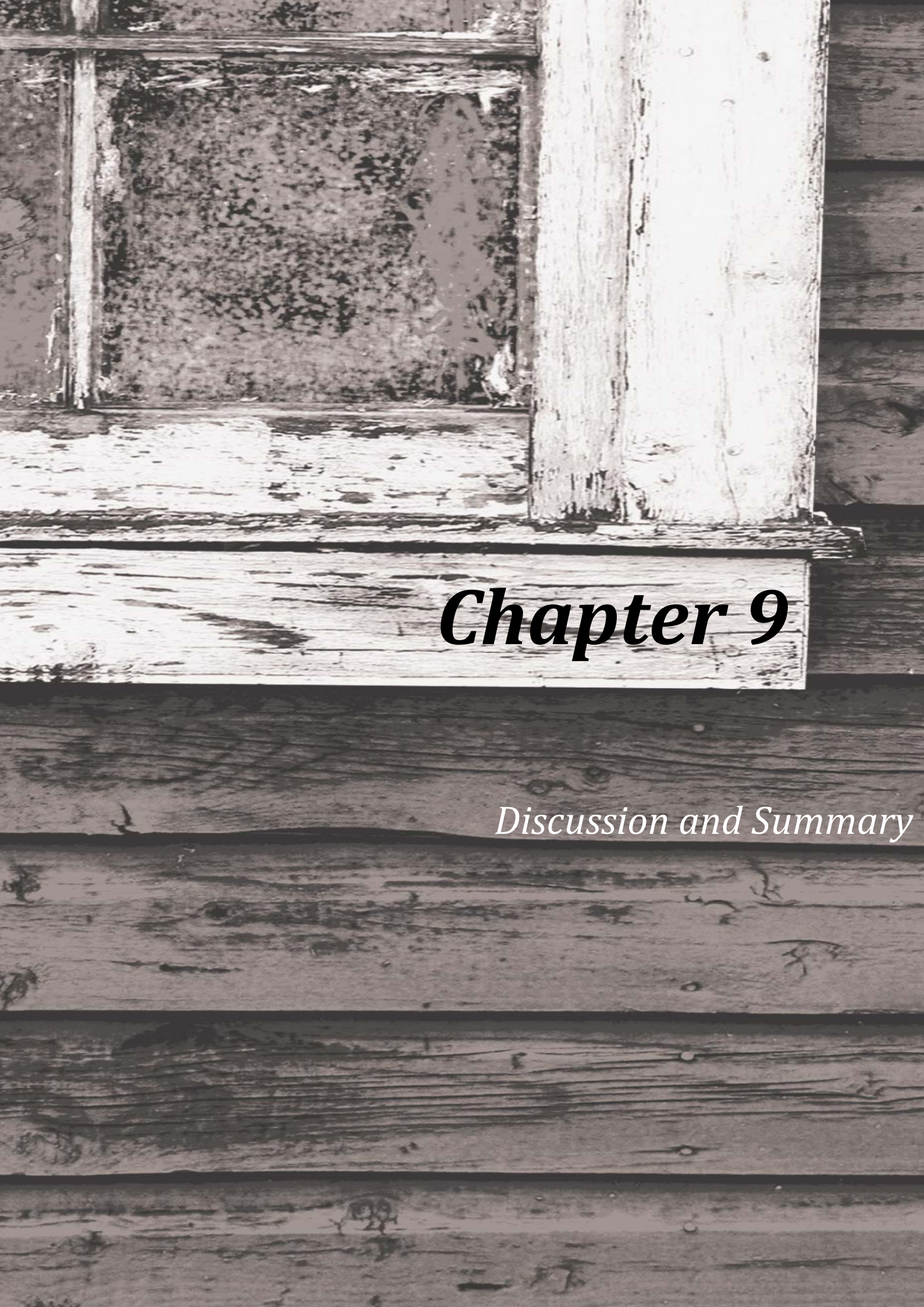


Chapter 9

Discussion and Summary

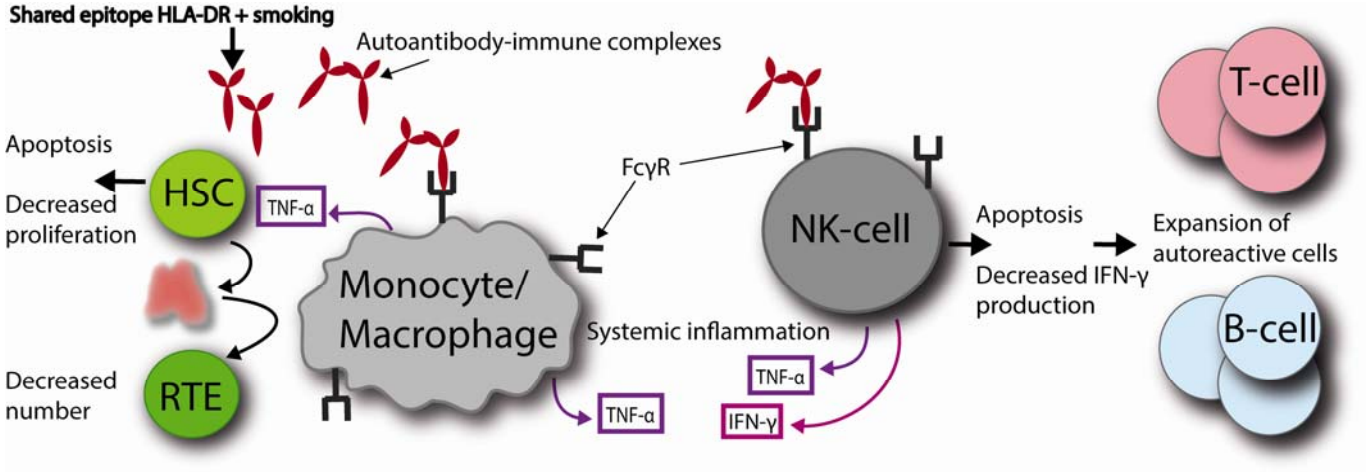
The main aim of the studies described in this thesis was to identify immune alterations characterizing the early stages of rheumatoid arthritis development. Integration of the data obtained by us with data from studies by others contribute to an improved understanding of the causes of RA from an immune perspective. The picture of the events leading to RA development, however, seems to be more clear for seropositive RA than for seronegative RA. RA patients seronegative for ACPA and RF represent a significantly smaller group. As autoantibodies play a major role in the immune alterations contributing to development of seropositive RA, the mechanistic outline pertains to seropositive RA only (**Fig.1**).

Development of seropositive RA

It has been suggested that the initial step in the development of seropositive RA is the emergence of systemic autoimmunity associated with RA, which refers to the emergence of ACPA and/or RF in the peripheral blood. This phenomenon has been attributed to the ill-defined interaction between the genetic (shared epitope-containing HLA-DR alleles) and environmental (smoking) factors; leading to the formation of neoepitopes by citrullination of proteins (1, 2). The presence of autoantibodies has been associated with the upregulation of various inflammatory markers in the peripheral blood. In line with other studies (3-5), we propose a functional role of autoantibodies in the induction of pro-inflammatory cytokine expression. Until now, in vitro studies demonstrated autoantibody-mediated induction of TNF-α and IFN-γ by monocytes and macrophages (3-5). IgG-containing autoantibodies, in the form of immune complexes, bind to immune cells expressing Fc receptors for IgG (FcγR). These cells include monocytes/macrophages (expressing FcγRI, FcγRIIA, FcγRIIC, FcγRIIIA) and NK-cells (expressing FcγRIIC, FcγRIIIA) (6). While binding of autoantibody-immune complexes to FcγR was shown to stimulate cytokine expression by both monocytes/macrophages and NK cells (4, 5, 7-9), these immune complexes may also trigger the apoptosis of NK-cells (8, 10-12). The systemic consequences of the ACPA/RF presence are the upregulation of pro-inflammatory cytokines and a drop in NK-cell numbers (**Chapter 3 and 4, this thesis**). These immune alterations may affect the process of RA development in several ways. The systemic increase of TNF-α enhances apoptosis and inhibits the proliferation of hematopoietic stem cells (HSC) (13), representing the progenitors of all immune cell types. This results in a decrease of HSC in the bone marrow and the periphery, a decrease of recent thymic emigrants (T-cells) and eventually in reduced numbers of circulating naive T-cells (14-17). In contrast, differentiated effector T-cells, i.e. CD161-expressing CD4+ T-cells, were found increased in seropositive arthralgia patients (**Chapter 5, this thesis**), regarded as an early stage of RA development. TNF-α, a key cytokine in RA pathogenesis, stimulates expression of various pro-inflammatory cytokines (i.e. IL-1β, IL-6) and chemokines (i.e. MCP-1), through the activation of NF-κβ (18-20). Augmentation of the pro-inflammatory phenotype, significant for the process of RA development, may be dependent on the concomitant presence of IL-17. Combination of TNF-α and IL-17 had profoundly greater effect on the

expression of cytokines, pro-thrombotic and pro-coagulant factors than either of these cytokines alone (20). IL-17 and IL-17-producing Th cells are implicated in the early stages of RA development (21, 22). Decrease of NK-cells, particularly CD56^{dim} NK-cells, may allow for an uncontrolled expansion of pro-inflammatory T-cells, as NK-cells play an immunosurveillant role against cells with tissue-destructive properties (23, 24). Furthermore, a direct role in killing autoreactive T-cells and B-cells by NK-cells has been demonstrated (25). At this initial stage of RA development (SAP) immune alterations are present at the level of peripheral blood, while the joints are not (yet) affected. This notion is supported by the data showing lack of signs of inflammation or increased immune cell infiltration within the synovium of SAP (26, 27).

Figure 1. Schematic depiction of the immune alterations induced by autoantibodies (ACPA or RF immune complexes) in seropositive RA patients.



Development of seronegative RA

Events preceding the development of seronegative RA are ill-defined and there is no consensus on systemcic or synovial markers discriminating between SP and SN RA. Results presented in this thesis revealed clear immune alterations that may be specific for seronegative RA. These include increased numbers of CD56^{bright} NK-cells (**Chapter 4, this thesis**), increased systemic levels of IL-10 and decreased systemic levels of Eotaxin (**Chapter 3, this thesis**). Due to the scarcity of available data, the role of these alterations in seronegative RA development remains to be investigated.

Characteristics of late-stage RA

Additionally, data presented in this thesis may serve to increase our understanding not only of the events in early stages of RA development, but also of alterations involved in maintaining local inflammatory processes in long-standing RA. When compared with healthy subjects, the peripheral blood of long-standing RA patients showed increased numbers of effector T-cells with pro-inflammatory functions. Several different populations of T-cells have been indentified. Despite the significant likelihood that these

populations show a phenotypical and functional overlap, and may originate from a common precursor population, this has not been assessed. Such T-cell populations, enriched in RA, include CD4+CD70+ T-cells, showing potent IFN-γ and IL-17 expression (28), Th17 cells (29), Th1 cells or cytotoxic and IFN-γ-producing CD28- T-cells (17). As to the question of a common precursor, we here demonstrated (**Chapter 5, this thesis**) a pathogenic role of precursor Th17 cells, defined as CD4+CD161+ T-cells, switching to non-classical Th1 cells at the level of the joint (likely by local IL-12 skewing (30)). Furthermore, our data suggest that the ligand for CD161, namely lectin-like transcript 1 (LLT1), expressed by macrophages in the RA synovium, putatively providing a co-stimulatory signal contributing to the Th1 phenotype, may be involved in the modulation of the pathogenic T-cell response (**Chapter 6, this thesis**).

SAP progression to RA

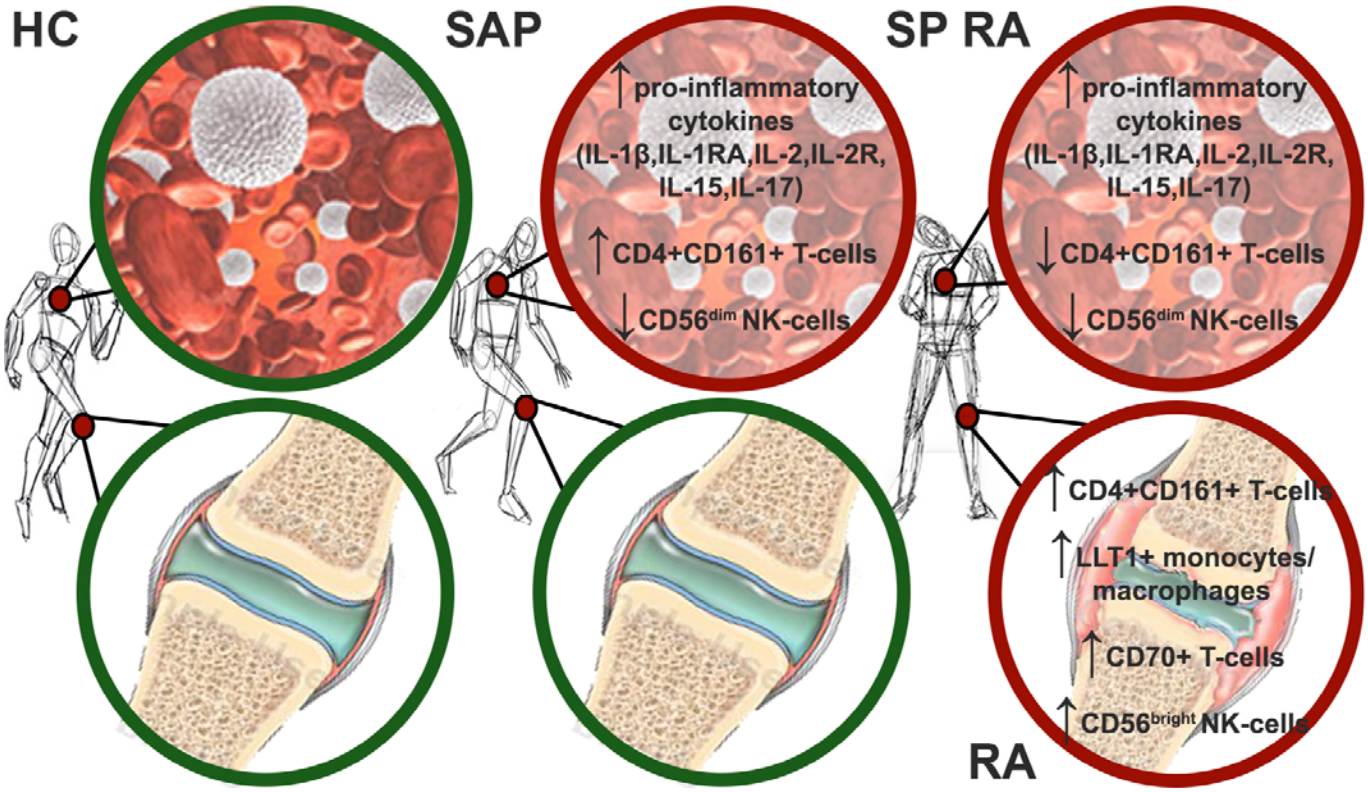
Events responsible for the expansion of the pathogenic immune response from the periphery to the joints, which underlie the progression to classifiable RA, are still subject of speculations. Increase of ACPA levels, broader ACPA specificity (epitope spreading) (3) and concomitant increase of multiple inflammatory markers in the peripheral blood (3, 31) have been associated with the switch from the pre-RA stage to full-blown RA. By comparing various immune markers in the periphery of at-risk patients (SAP) who progressed to RA during the follow-up and SAP who do not progress, we have identified cytokines that may be involved in the switch to RA. These include increased systemic levels of IL-5, MIP-1β, IL-1RA and IL-12; among which the best ability to predict RA development was found for IL-5 (**Chapter 3, this thesis**). Furthermore, we observed modulation of circulating CD4+CD161+ T-cells, characterized by a potent IFN-γ and IL-17 producing capacity, in SAP and newly diagnosed RA patients. The data obtained showed elevated frequencies of these cells in SAP but decreased frequencies in early RA, thereby suggesting the migration of pro-inflammatory CD4+CD161+ T-cells to the joints which may contribute to RA development (**Chapter 5, this thesis**). Our data also revealed a delay in the downregulation of CD70 expression in peripheral T cells from both SAP and early RA patients (**Chapter 7, this thesis**). Prolonged expression of an important co-stimulatory molecule on pro-inflammatory T-cells may contribute to increased autoreactivity.

In summary (**Fig.2**), we have:

- identified immune alterations involved in the development of seropositive RA (decline of NK-cells; increased systemic levels of pro-inflammatory cytokines, i.e. IL-1β, IL-1RA, IL-2, IL-2R, IL-15, IL-17);
- revealed novel systemic immune alterations characterizing seronegative RA (increase of $CD56^{bright}$ NK-cells, increase of IL-10, decrease of Eotaxin levels)

- added novel data about immune alterations that contribute to local inflammation and joint destruction at later stages of RA (expression of the ligand for CD161, LLT1, by synovial macrophages; accumulation of CD4+CD70+ T-cells and CD4+CD161+ T-cells in the synovial fluid and synovial tissue).
- identified immune markers in SAP that may help to identify high-risk (for progression to RA) patients (i.e. IL-5, MIP-1β, IL-1RA, IL-12)

Figure 2. Schematic summary of results described in this thesis, including the immune alterations and their localization in the studied cohorts.



Limitations and future perspectives

In this thesis we investigated the immune profiles in different phases of RA in a cross-sectional design. Although this can help in characterizing the different phases of RA, future studies should preferably be done in a longitudinal design. That would allow to more directly study cause-effect relationships within the context of the same individual. Clearly, this can only be done if basic research is combined with strict clinical monitoring.

Some of the markers in our studies were validated in independent cohorts. For the majority of the studied immune markers, however, we would recommend further validation in independent patient cohorts.

Our studies on differences in SP vs SN RA underline the importance of stratifying RA patients according to the autoantibody status in studies investigating pathological pathways involved in RA and also in the designs of clinical trials.

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Appendices

Nederlandse samenvatting

Acknowledgements

Curriculum Vitae

List of publications

Nederlandse samenvatting

In dit proefschrift beschrijven we onze zoektocht naar immunologische bloed merkers die de vroege stadia van reumatoïde artritis (RA) karakteriseren. Goede merkers zijn van belang in de vroege herkenning van hoog risico patiënten en maken een verbeterde behandeling mogelijk. In onze studies zijn de volgende patiëntengroepen geincludeerd: i) seropositieve artralgie patiënten (SAP), een groep met een hoog risico (~30%) op het ontwikkelen (binnen 1-2 jaar) van RA en ii) recent gediagnosticeerde RA patiënten die alleen behandeld worden met niet-steroïdale anti-inflammatoire geneesmiddelen (NSAID). De RA patiënten kunnen nog worden onderverdeeld in seropositieve en seronegatieve patiënten (resp. afgekort als SP RA en SN RA) waarbij SP RA patiënten autoantistof (ACPA en/of reuma factor (RF)) positief zijn. Gezonde donoren van een vergelijkbare leeftijd werden bestudeerd om ziekte markers te identificeren.

Omdat het voorkomen van RA toeneemt met ouder worden hebben we in **hoofdstuk 2** onderzocht of er bewijs is voor versnelde veroudering van het immuun systeem (immunosenescence) in RA. Onze conclusie was dat de versnelde veroudering van het immuun systeem in RA patiënten waarschijnlijk het gevolg is van de ontsteking in deze ziekte.

De eerste stap in de ontwikkeling van seropositieve RA is geassocieerd met de vorming van autoantistoffen gericht tegen gecitrulineerde eiwitten, ook wel aangeduid met ACPA, en RF die kunnen worden aangetroffen in het bloed. De aanwezigheid van auto-antilichamen is geassocieerd met de toename van meerdere onstekingbevorderende stoffen (cytokinen) in het bloed. In **hoofdstuk 3** hebben we dan ook het cytokine profiel van SAP, SP RA en SN RA onderzocht. Tevens hebben we onderzocht of we hoog risico SAP patiënten kunnen identificeren op basis van het cytokine profiel bij de eerste bloedafname. Onze resultaten laten zien dat het cytokinen profiel van SAP en SP RA sterke overeenkomsten vertonen. Daarentegen zijn de cytokine profielen van SP en SN RA duidelijk verschillend. De data suggereren dat de autoantistoffen een functionele rol spelen in de inductie van ontstekingsbevorderende cytokinen. Een verhoogde serum IL-5 spiegel in SAP lijkt geassocieerd met de ontwikkeling van RA. Of deze merker daadwerkelijk voorspellende waarde heeft zal moeten blijken in vervolgstudies.

De overeenkomsten tussen SAP en SP RA komen ook naar voren in **hoofdstuk 4** waar we de mogelijke rol van NK cellen hebben onderzocht. In zowel SAP als ook SP RA zien we een afname van de $CD56^{dim}$ (cytotoxische) NK cellen. Daarentegen zien we in SN RA juist een toename van de $CD56^{bright}$ (immuunregulatoire) NK cellen. De afname van de $CD56^{dim}$ NK cellen in SAP en SP RA wordt waarschijnlijk veroorzaakt door ACPA/RF immuun complexen die binden aan de Fc receptor CD16; CD16 komt verhoogd tot expressie op $CD56^{dim}$ NK cellen. In **hoofdstuk 4** laten we zien dat synthetische immuuncomplexen inderdaad leiden tot apoptose van $CD56^{dim}$ NK cellen, maar niet van $CD56^{bright}$ NK

cellen, in vitro. Tevens zien we dat synthetische immuuncomplexen ontstekingsbevorderende cytokinen induceren in deze subset van NK cellen. De afname van de $CD56^{dim}$ NK cellen die in staat zijn om autoreactieve witte bloedcellen te elimineren, zou een mogelijk mechanistisch verklaring kunnen zijn in de vroege ontwikkeling van RA.

Op grond van de bevindingen in hoofdstuk 3 en 4, en op basis van recent gepubliceerde bevindingen van anderen, kan geconcludeerd worden dat auto-antilichamen een functionele rol spelen bij het ontwikkelen van RA en dus niet alleen diagnostische merkers zijn.

Omdat helper T cellen een rol spelen in de pathogenese van RA hebben we in **hoofdstuk 5** meer specifiek onderzoek gedaan naar de rol van Th17 voorlopercellen in SAP en RA. In vergelijking met gezonde controles vonden we in het bloed van SAP een toename van Th17 voorlopercellen, gekarakteriseerd op basis van de expressie van CD4 en CD161. In het bloed van nieuw gediagnosticeerde RA patiënten, voordat deze werden behandeld met immuun modulerende geneesmiddelen, zagen we een afname van deze cellen. Behandelen met MTX zorgde voor een afname van de DAS28 en een normalisering van de perifere Th17 voorlopercellen. Th17 voorlopercellen werden aangetroffen in het synovium en in het synoviaal vocht. In synoviaal vocht bleken deze cellen echter te switchen naar een Th1 fenotype. Studies van anderen hebben laten zien dat deze switch tot stand kan worden gebracht door lokale productie van IL-12. De rol van Th17 voorlopercellen en de mogelijke interactie van deze cellen met lokale antigeen presenterende cellen werd onderzocht in **hoofdstuk 6**. De natuurlijke ligand van CD161 is lectin-like transcript 1 (LLT1). Dit molecuul werd aangetoond op macrofagen in het synoviale weefsel van RA patiënten. De interactie tussen CD161+ T cellen en LLT1+ antigeen presenterende cellen zou tot lokale co-stimulatie kunnen leiden en daarmee kunnen bijdragen aan de immunopathologie van RA. In **hoofdstuk 7** hebben we het fenotype van CD70+ T cellen nader gekarakteriseerd. CD70 vormt met CD27 ook een co-stimulatoir receptor paar. CD70+ T cellen bleken actief prolifererende effector T cellen. In RA werden deze cellen ook op het nivo van het gewricht aangetoond. Opvallend was dat de kinetiek van CD70 expressie verstoord leek in SAP en in RA (vertraagde downregulatie). In **hoofdstuk 8** bespreken we de rol van immuunveroudering in relatie tot chronische ontsteking; een van de kenmerken van autoimmuun-gemedieerde aandoeningen zoals RA.

In **hoofdstuk 9** bespreken we de belangrijkste bevindingen en de implicaties hiervan voor ons begrip van de vroege processen in RA ontwikkeling.

- Het immuun profiel van SAP en SP RA is vergelijkbaar. Daarentegen is het immuun profiel van SN RA duidelijk anders.
- Hoewel T cellen betrokken zijn bij de immunopathogenese van RA lijken het geen goede perifere merkers te zijn voor de vroege fase van RA.

- In de latere fase van RA en op de plaats van ontsteking zien we een toename van het aantal CD4+ T-cellen met CD161 expressie, een toename van CD4+ T-cellen met CD70 expressie en monocyten/ macrofagen met LLT1 expressie. Deze bevindingen ondersteunen de gedachte dat een T-cel gemedieerde immuunrespons een belangrijke rol speelt in het lokale ontstekingsproces bij RA.
- IL-5 is een mogelijke merker in de vroege herkenning van hoog risico SAP patiënten.

De immunologische verschillen tussen SP RA en SN RA patiënten wijzen naar de betrokkenheid van verschillende pathologische routes in deze aandoening en suggereren de noodzaak om bij basaal onderzoek en klinische studies RA patiënten in te delen naar auto-antilichaam status.

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Curriculum Vitae

Paulina Chałan was born on 25th of August 1985 in Proszowice, Poland. She completed her secondary education at Jan Śniadecki High School No.2 in Kielce. In the years 2004-2009 she studied biotechnology at the University of Warsaw, Faculty of Biology. Her bachelor's degree project, entitled "Optimalisation of the conditions of in vitro culture of Spodoptera littoralis sperm bundles", was conducted in the Department of Animal Physiology, under supervision of Ewa Joachimiak, PhD. Her master's degree research project was conducted in the Department of Immunology, under supervision of Prof. Grażyna Korczak-Kowalska (promotor) and Anna Korecka, PhD (supervisor). The aim of the project was to study the age-dependent effects of immunosuppressive drugs- cyclosporine A and rapamycin on populations of human T cells, defined phenotypically as CD4+CD25+ and CD8+CD28- (using two groups of donors: young vs. old). She defended her master's thesis in 2009. During her studies, she participated in the summer internship programme, supported by Erasmus Programme, in the laboratory of Prof. Maria Bokarewa at the Department of Rheumatology and Inflammation Research, Sahlgrenska Medical Academy, Göteborg University, Sweden. During its framework she studied expression of the enzyme ADAMTS-13 in the blood and synovial fluid of patients with rheumatoid arthritis.

In October 2009 Paulina started PhD studies at the University of Groningen, University Medical Center Groningen, The Netherlands. She joined the Department of Rheumatology and Clinical Immunology and conducted her PhD research project under the supervision of Prof. Annemieke M.H. Boots (promotor), Liesbeth Brouwer, MD, PhD and Bart-Jan Kroesen, PhD (supervisors). Her main research interest focused on alterations of systemic immune response (innate and adaptive) in the early stages of rheumatoid arthritis development.

List of publications

1. Chalan P, Bijzet J, van den Berg A, Kluiver J, Kroesen BJ, Brouwer E, Boots AMH. Serum immune markers discriminate between seropositive and seronegative rheumatoid arthritis and identify seropositive arthralgia patients at risk to progress to rheumatoid arthritis. *Submitted.*

2. Chalan P, Bijzet J, Kroesen BJ, Brouwer E, Boots AMH. Altered NK-cell subsets in seropositive arthralgia and early rheumatoid arthritis patients are associated with autoantibody status. *Submitted.*

3. Chalan P, van den Berg A, Kroesen BJ, Brouwer E, Boots AMH. Rheumatoid Arthritis, Immunosenescence and the Hallmarks of Aging. *Current Aging Science 2015;8(2):131-46.*

4. Chalan P, Bijzet J, Huitema M, Kroesen BJ, Brouwer E, Boots AMH. Expression of Lectin-Like Transcript 1, the ligand for CD161, in Rheumatoid Arthritis. *PLoS One 2015 Jul 6;10(7):e0132436.*

5. Park JK, Han BK, Park JA, Woo YJ, Kim SY, Lee EY, Lee EB, **Chalan P**, Boots AM, Song YW. CD70-expressing CD4 T cells produce IFN-γ and IL-17 in rheumatoid arthritis. *Rheumatology 2014 Oct;53(10):1896-900.*

6. van der Geest KS, Abdulahad WH, **Chalan P**, Rutgers A, Horst G, Huitema MG, Roffel MP, Roozendaal C, Kluin PM, Bos NA, Boots AM, Brouwer E. Disturbed B cell homeostasis in newly diagnosed giant cell arteritis and polymyalgia rheumatica. *Arthritis & Rheumatology 2014 Jul;66(7):1927-38.*

7. Chalan P, Kroesen BJ, van der Geest KS, Huitema MG, Abdulahad WH, Bijzet J, Brouwer E, Boots AM. Circulating CD4+CD161+ T lymphocytes are increased in seropositive arthralgia patients but decreased in patients with newly diagnosed rheumatoid arthritis. *PLoS One 2013 Nov 1;8(11):e79370.*

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